

Biomaterial Control of Therapeutic Stem Cells

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Biomaterial Control of Therapeutic Stem Cells

By

Akon Higuchi

National Central University, Taiwan

Email: higuchi@ncu.edu.tw



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Preface

This book, *Biomaterial Control of Therapeutic Stem Cells*, describes biomaterials for culture and differentiation of human embryonic stem (hES) cells and human induced pluripotent stem (hiPS) cells as well as biomaterials used in clinical trials of stem cell therapy. Human pluripotent stem (hPS) cells, which cover both hES cells and hiPS cells, show promise for drug discovery and regenerative medicine applications. These stem cells cannot be cultured on conventional tissue culture plates in general but on biomaterials that have specific interactions with the hPS cells. Differentiation is regulated by the biological and physical cues conferred by the biomaterials. This book provides a systematic discussion of these topics, bridging the gap between fundamental biomaterials research of stem cells and their use in clinical trials. The book is mainly written for material scientists, whether or not they have any knowledge of stem cells, but who are eager to begin their research into stem cell culture and differentiation. The motivation to write this book originates with my research history. I started my research in the field of physical chemistry (*i.e.*, transport of small molecules through polymeric membranes) when I was an undergraduate student under the guidance of Professor Toru Kawai at the Tokyo Institute of Technology, Japan. Membrane research was a hot topic in the 1970s, but the research boom in membrane science quickly diminished after 1970. I always wanted to work on such hot topics but I had no knowledge of organic chemistry or biochemistry at that time, which prevented me from continuing my bioengineering research. Fortunately, I learned about the simple chemical reactions of polymerization and surface modification of materials during my work experience at a private chemical company (Asahi Kasei Corporation) in 1985, and acknowledge the help and support of my colleague, Mr Kubota Noboru, and my supervisor, Mr Takayuki Yokoyama, during my time there. I then began my own research at Meiji University, Japan. I started my research targeting the bio-field using

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materials such as biosensor preparation, but wanted to move on from the area of biosensor research. When I set up my own laboratory at Seikei University, Tokyo, in 1993, Dr Seiji Manabe kindly supported me and we worked together on transport phenomena through virus removal filters (Planova). I was then fortunate to learn the techniques of cancer cell culture from Dr Manabe's laboratory. From 1990 to 2010, I worked on the cultivation of hematopoietic stem cells and adipose-derived stem cells on several biomaterials. Several doctors from a variety of clinics kindly supplied peripheral blood and umbilical cord blood, including Dr Mitsuo Hattori from Seikei University. Collaboration with doctors is very important to progress in this field. Myself and the students in my laboratory isolated stem cells from peripheral blood, umbilical cord blood and fat tissues and cultured these stem cells on home-made biomaterials. I moved from Seikei University in Tokyo, Japan, to the National Central University, Taiwan, in 2007. Since 2010, my laboratory has been more focused on hES cell and hiPS cell culture and differentiation, which are cultured on several biomaterials. It is challenging to control the differentiation fate of stem cells by regulating their micro-environment, such as by controlling only the physical or chemical properties of biomaterials. However, we believe that we can regulate the stem cell fate of differentiation by cell culture biomaterial characteristics, which are micro-environments of stem cells. This issue is also significant in terms of human society; our performance and ability, including our social position and status, could be improved by our environment, which is not controlled solely by our heredity. We think that the role of the stem cell microenvironment (stem cell culture biomaterials) in deciding and regulating stem cell fate of differentiation is similar to that of the human fate in our society. Therefore, any material scientists who are interested to progress their research in the stem cell field can gain fundamental knowledge as well as the current status of stem cell culture and differentiation, and current trends in stem cell therapy from this book. I am happy for readers to use this book as a dictionary, which includes fundamental data such as three germ layer gene and proteins, extracellular matrices (ECMs) and ECM-derived oligopeptide, integrin binding for specific extracellular matrix, research examples of stem cell culture using specific extracellular matrices, differentiation methods into specific and desired lineage of the cells, and recent clinical trial examples, *etc.*

Finally I would like to thank the Royal Society of Chemistry for giving me a chance to write this book, especially Michelle Carey and Connor Sheppard. I also thank my colleagues, Shang-Lin (Sophie) Chen, Dr Tzu-Cheng Sung, Professor Yung Chang, Professor Qing-Dong Ling, Professor Akihiro Umezawa, other friends and my family members. I also thank Asahi Kasei Corporation, Meiji University, Seikei University, National Central University, King Saud University, Riken, National Center for Child Health and Development, Ministry of Education, Culture, Sports, Science, and Technology of Japan, and Ministry of Science and Technology, Taiwan for supporting my research.

Akon Higuchi

Abbreviations

2D	two-dimensional
3D	three-dimensional
ADS cell, ADSC	adipose-derived stem cell
AEtMA-Cl	2-(acryloyloxyethyl) trimethylammonium chloride
AFM	atomic force microscopy
AFP	α -fetoprotein
Akt	serine–threonine kinase
ALP	alkaline phosphatase
AMI	acute myocardial infarction
AMS cell	amniotic membrane-derived stem cell
APMAAm	aminopropylmethacrylamide
ASGPR	asialoglycoprotein receptor
BBB	Basso, Beattie, Bresnahan
BBS	borate buffered saline
BIO	bromo-indirubin-3'-oxime
BM	bone marrow
BMN cell	bone marrow mononuclear cell
BMS cell	bone marrow-derived stem cell
BSA	bovine serum albumin
CA	cellulose acetate
CABG	coronary artery bypass grafting
CALP	h1-calponin
CDC	cardiosphere-derived cell
CDS	chondroitin sulfate
cGMP	current good manufacturing practice
CM	condition medium
CMP	collagen mimetic oligopeptide
CNS	central nervous system

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COL	collagen
CS	chitosan
CSC	cardiac stem cell
CV	conduction velocity
CXCR4	chemokine receptor type 4
DA cell	dopaminergic cell
DE	definitive endoderm
DEAEA	2-(diethylamino)ethyl acrylate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DPS cell	dental pulp stem cell
EB	embryoid body
EC	endothelial cell
ECM	extracellular matrix
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
EHS sarcoma cell	Engelbreth-Holm-Swarm sarcoma cell
ELP	elastin-like polypeptide
ERK	extracellular signal regulated kinase
ES cell, ESC	embryonic stem cell
ESV	end-systolic volume
ETDRS	early treatment diabetic retinopathy study
EV	extracellular vesicles
EVAL	poly(ethylene-co-vinyl alcohol)
FA	focal adhesion
FACS	fluorescence activated cell sorting
FAK	focal adhesion kinase
FBS	fetal bovine serum
FG	fibrinogen
FGF	fibroblast growth factor
FISH	fluorescence <i>in situ</i> hybridization
FN	fibronectin
FOXA2	forkhead box A2
GABA inhibitory cell	gamma-aminobutyric acid inhibitory cell
GAG	glycosaminoglycan
G-CSF	granulocyte colony-stimulating factor
GDNF	glial cell-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GMP	good manufacturing practice
HA	hyaluronic acid
H&E	hematoxylin and eosin
HBP	heparin-binding peptide
HUVEC	human umbilical vein endothelial cell
HYA	hyaluronic acid
hADS cell	human adipose-derived stem cell
hAS cell	human adult stem cell
hBMN cell	human bone marrow mononuclear cell

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hBMS cell	human bone marrow stem cell
hCNS-SC	human central nervous system stem cell
hES cell, hESC	human embryonic stem cells
hMS cell	human mesenchymal stem cell
HNF1 α	hepatocyte nuclear factor 1 homeobox A
hiPS cell, hiPSC	human induced pluripotent stem cell
hPS cell, hPSC	human pluripotent stem cell
hWJ-MS cell	human Wharton's jelly-derived mesenchymal stem cell
ICC	inverted colloidal crystal
ICG	indocyanine green
IgG	immunoglobulin
ILK	integrin-linked kinase
INL	inner nuclear layer
IPC	insulin producing cell
IPL	inner plexiform layer
IPN	interpenetrating network
iPS cell	induced pluripotent stem cell
KSR	knockout serum replacement
LCST	low critical solution temperature
LDL	low-density lipoprotein
LIF	leukemia inhibitory factor
LLS	linear local shortening
LN	laminin
LV	left ventricular
LVEDV	left ventricular end-diastolic volume
LVEF	left ventricular ejection fraction
LVESV	left ventricular end-systolic volume
MACS	magnetic-activated cell sorting
MAPK	mitogen-activated protein kinases
MARC chip	multi-architecture chip
MC	microcarrier
MCFU	mesenchymal colony-forming unit
MEF-CM or MEFs-CM	condition medium from MEF(s)
MEF2c	myocyte enhancer factor 2C
MFB	medial forebrain bundle
MI	myocardial infarction
MLC-2a	myosin light chain-2a
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
MS cell, MSC	mesenchymal stem cell
mES cell	mouse embryonic stem cell
NCM	neonatal rat cardiomyocyte
NCSC	neural crest stem cell
NHS	<i>N</i> -hydroxysuccinimide
NPC	neural progenitor cell

NPS cell	neural progenitor/stem cell
NS cell	neural stem cell
OCT	optical coherence tomography
OmpA	outer membrane protein A
ONL	outer nuclear layer
PA	oligopeptide amphiphile
PAAm, PAA	polyacrylamide
PAS	biologically active peptides
PBS	phosphate buffered saline
PCBMA	poly(carboxybetaine methacrylate)
PCI	percutaneous coronary intervention
PCL	poly(3-caprolactone)
PDDL	poly-DL-lactic acid
PDL	poly-D-lysine
PE	polyethylene
PE cell	pancreatic endoderm cell
PECM	placenta-derived ECM scaffolds
PEG	polyethylene glycol
PEGDA	poly(ethylene glycol) dimethacrylate
PEODA	poly(ethylene oxide) dimethacrylate
PET	positron emission computed tomography or poly(ethylene terephthalate)
PF	posterior foregut
PGA	poly(glycolic acid)
PGT	primitive gut tube
PHA	polyhydroxyalkanoate
PhaC	PHA synthase
PhaP	PHA granule-associated proteins
PHBHHx	poly(3-hydroxybutyrate-co-hydroxyhexanoate)
PHEMA	poly(2-hydroxyethyl methacrylate)
PLCL	poly(L-lactic acid)-co-poly(3-caprolactone)
PLGA	poly(lactic-co-glycolic acid)
PLL	poly-L-lysine
PMEDSAH	poly(2-[methacryloyloxy]ethyl dimethyl-[3-sulfopropyl] ammonium hydroxide)
PMVE- <i>alt</i> -MA	poly(methylvinyl ether- <i>alt</i> -maleic anhydride)
PNIPAM	poly(<i>N</i> -isopropylacrylamide)
PPEGMA	poly(poly[ethylene glycol] methyl ether methacrylate)
PMETAC	poly([2-(methacryloyloxy)ethyl])
PPS	phosphatidylserine
PROD	pentoxeresorufin o-dealkylase activity
PS	polystyrene
PSC, PS cell	pluripotent stem cell
PSPMA	poly(3-sulfopropyl methacrylate)
PVA	polyvinyl alcohol

Abbreviations

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PVC	premature ventricular contraction
PVDF	polyvinylidene fluoride
pDTEc	poly(desaminotyrosyltyrosine ethylestercarbonate)
RCS rat	Royal College of Surgeons rat
RGC	retinal ganglion cell
RPE	retinal pigment epithelium
rVN	recombinant vitronectin
SAM	self-assembled monolayer
SDF-1 α	stromal cell-derived factor-1 α
SD-OCT	spectral domain optical coherence tomography
SERCA2a	sarco/endoplasmic reticulum Ca ²⁺ -ATPase
Shh, SHH	sonic hedgehog
SIS	small intestinal submucosa
SMC	smooth muscle cell
SM2	smooth muscle myosin heavy chain
SPECT	single-photon emission computed tomography
SR	serum replacement
SSEA-4	glycolipid stage-specific embryonic antigen 4
sGAG	sulphated glycosaminoglycan
TCP	tissue culture polystyrene
TH	tyrosine hydroxylase
Tra-1-81	tumor rejection antigen 1-81
UC	urothelial cell
USC	urine-derived stem cell
VCM	ventricular cardiomyocyte
VDP	vitronectin-derived peptide
VEGF	vascular endothelial growth factor
VN	vitronectin
VTa	ventral tegmental area
WJ-MS cell	Wharton's jelly-derived mesenchymal stem cell

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CHAPTER 1

Introduction

1.1 Introduction

Each year, millions of people suffer from spinal cord injury and diseases such as myocardial infarction, diabetes, and leukemia. In the past, therapeutic approaches have been limited to the removal of injured parts by surgery and medical treatment.

Human pluripotent stem (hPS) cells have high differentiation ability relative to adult stem cells such as adipose-derived stem cells and bone marrow-derived stem cells. Several studies have demonstrated that hPS cells can be differentiated into specific cell lineages derived from three germ layers.^{1,2} Thus, hPS cells are a promising source for the replacement of damaged or lost cells in regenerative medicine. However, it is necessary to control the proliferation and differentiation of stem cells in xeno-free culture conditions for the clinical use of stem cells. In this case, cell culture biomaterials play an important part in the stem cell fate of proliferation as well as the stem cell fate of differentiation into specific lineages of cells that are going to be used for drug discovery and regenerative medicine.

1.2 Stem Cells

Stem cells are capable of self-renewal, proliferation, and differentiation to various cell lineages, making them advantageous for regenerative medicine applications. Importantly, self-renewal and cellular proliferation are not synonymous, because the former term encompasses both the differentiation and future mitotic potential of the daughter cells in addition to cell division.^{3–5}

Depending on the type and maturity of stem cells in the tissue, stem cell potency and capacity for self-renewal can be varied.⁶ There are two main types of stem cells, embryonic and non-embryonic cells. Embryonic stem

(ES) cells are pluripotent and ES cells can differentiate into the cells derived from all three germ layers (ectoderm, mesoderm, and endoderm). Non-ES cells are multipotent. Their potential to differentiate into different cell types seems to be more limited³ and more importantly, multipotent stem cells have an aging problem (limited proliferation) which ES cells do not have (infinite proliferation). The differentiation potential can be classified into four levels: totipotent, pluripotent, multipotent, and unipotent stem (progenitor) cells (Figure 1.1).⁷

Totipotent stem cells can differentiate into embryonic and extra-embryonic cell types, which can construct a complete and viable organism. Totipotent cells are produced from the fusion of an egg and a sperm cell. The fertilized egg and the cells produced by the first few divisions of the fertilized egg are totipotent. Totipotent stem cells give rise to somatic stem/progenitor cells and primitive germ-line stem cells.⁸

Pluripotent stem cells can differentiate into nearly all cell types of the adult organism, because they have the ability to differentiate into the cells derived from three germ layers: endoderm, mesoderm, and ectoderm.^{9,10}

ES cells, which are pluripotent stem cells, are derived from totipotent cells of the early mammalian embryo and are capable of differentiating into cells representing the three embryonic germ layers, namely ectoderm, mesoderm, and endoderm or any of more than 100 cell types present in the adult body, and are characterized by self-renewal, immortality, and pluripotency. ES cells are unlimited and show undifferentiated proliferation *in vitro*.^{11–15}

Multipotent stem cells can differentiate into a number of cells, but only those of a closely related family of the cells. These are stem cells but can only differentiate into a limited number of cell types. For example, the bone marrow contains multipotent stem cells that give rise to all the cells of the

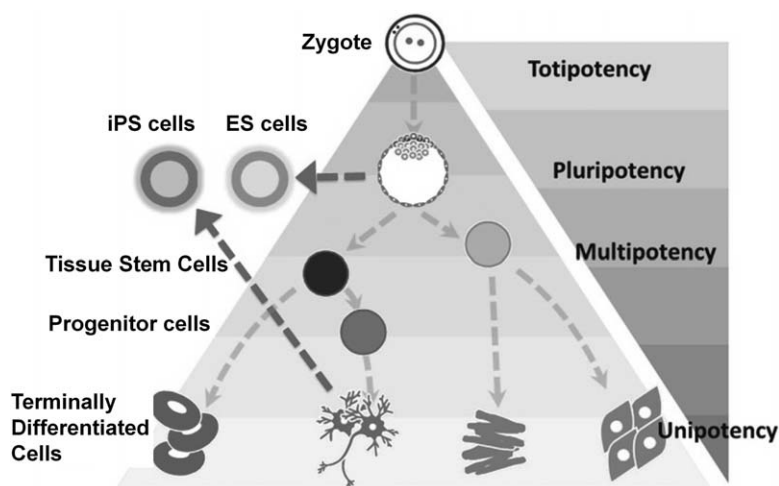


Figure 1.1 Hierarchical potential of stem cell development.⁷
Adapted from ref. 7 with permission from Springer Nature.

blood but not to other types of cells (hematopoietic stem cells), as well as bone marrow-derived stem cells, which are typical mesenchymal stem (MS) cells. Adipose tissue also contains a source of multipotent stem cells (adipose-derived stem cells).¹⁶

Unipotent stem (progenitor) cells denote a state lineage plasticity to differentiate into only a few cells.¹⁷ Unipotent stem cells are those such as lymphoid or myeloid stem cells. The corneal epithelium is a squamous epithelium¹⁸ that is constantly renewing and is regarded as an unipotent stem cell.¹⁹ Unipotent progenitor cells can produce only one cell type (their own), but have the property of self-renewal, which distinguishes them from non-stem cells. Most epithelial tissues self-renew throughout adult life due to the presence of unipotent progenitor cells.²⁰

There are ethical difficulties regarding the use of human embryos, as well as the problem of tissue rejection following transplantation in patients. One way to circumvent these issues is the generation of pluripotent cells directly from the patient's own cells.²¹ Somatic cells can be reprogrammed by transferring their nuclear contents into oocytes²² or by fusion with ES cells,^{23,24} indicating that unfertilized eggs and ES cells contain factors that can confer totipotency or pluripotency to somatic cells.

Through analyzing the gene expression profiles of ES cells, many highly expressed genes in ES cells have been identified. In 2006, Yamanaka and his colleague²¹ successfully introduced 24 transcription factors (pluripotent genes) that are highly expressed in ES cells into the fibroblast cells derived from fetal mice. Surprisingly, some ES-like colonies appeared in the culture dish within 2 weeks of retroviral infection. Moreover, these ES-like cells could be propagated *in vitro* and resemble ES cells morphologically after many cell passages.¹⁴ They found ultimately that four transcription factors including *Oct4*, *Sox2*, *Klf4*, and *c-Myc*²⁵ (nowadays, *Oct4*, *Sox2*, and *Klf4* or less transcription factor²⁶) were essential for converting the fibroblast cells into induced pluripotent stem (iPS) cells by reducing the factors one by one in the process of retroviral infection. The iPS cells were created by inducing the specialized cells to express genes that were normally present in ES cells and that control cell functions.²⁷ However, iPS cells could be successfully derived from the differentiated somatic cells simply based on the morphology changes and no genetic selection was needed, which indicated that human somatic cells without genetic modification could be reprogrammed successfully.^{28,29}

Since their discovery in the mid-2000s, newer generations of iPS cell lines have been created through various non-integrating reprogramming strategies, such as approaches using mRNA,³⁰ episomes,^{31,32} minicircles,³³ piggyBac transposons,³⁴ recombinant proteins³⁵ or Sendai virus.^{36,37}

In 2012, Shinya Yamanaka and Sir John Gurdon were awarded a Nobel Prize for their combined efforts in discovering that “mature cells can be reprogrammed to become pluripotent”. iPS cells were created by inducing the specialized cells to express pluripotent genes that were normally present in ES cells and that controlled cell functions. In addition, iPS cells have

advantages over ES cells: iPS cells are capable of generating autologous and non-immunogenic patient-specific therapies and can more easily provide cell-based disease models from genetically predisposed patients. These newer generations of iPS cell lines avoid the tumorigenicity risks associated with the genomic integration of reprogramming factors and are a powerful way of creating patient- and disease-specific cell lines for research.^{27,37}

1.3 The Extracellular Matrix

The extracellular matrix (ECM) is the extracellular part of animal tissues, which maintains structural back-up for the stem cells, as well as inspiring many key biological properties.³⁸ ECM proteins can ascertain whether stem cells will multiply or undergo growth retardation, differentiate or remain static, and expand or undergo apoptotic death (programmed death by cell).³⁹ Then, the ECM proteins are significant causes in recreating the biological roles of stem cells *in vitro* that help stem cells to induce into various heredities, for example, β -cells, hepatocytes, neural cells, cardiomyocytes, adipocytes, chondrocytes, and osteoblasts. The differentiation of stem cells in culture relies upon the origin, structure, components, and amount of ECMs that are used. Because ECMs are used as matrices or hydrogels for the arrangement of cells in tissues, ECMs are the major cell cultivation ingredients used to checkmate the differentiation and expansion of stem cells in regenerative medicine and translational medicine, both *in vivo* and *in vitro*. Therefore, we will discuss the differentiation of stem cells cultivated on materials composed of appropriate ECMs and on the chemical and biological contact between stem cells and ECMs in Chapter 2.

1.4 hPSC Culture on Biomaterials

Human PS (hPS) cells including human iPS (hiPS) cells^{40,41} and human ES (hES) cells^{42–44} have promise for drug discoveries, disease modeling, and regenerative medicine. In order to fully utilize hPS cells in tissue engineering and cell therapy, the advancement of a well-defined microenvironment for culturing hPS cells is needed.^{45–52} The present highest quality level for proliferation and maintenance of hPS cells is typically cultured on feeder cells (*e.g.*, mouse embryonic fibroblasts or human feeder layer cells) or on Geltrex^{53,54} and Matrigels.^{55–59} The use of feeder layers (cells) to cultivate hPS cells is a laborious process, which varies depending on the specific lots of feeder cells or skill of preparation of feeder cells. In contrast, Geltrex and Matrigel are made of molecules extracted from mice sarcomas of Engelbreth–Holm–Swarm mice. hPS cell culture on the coating dishes with Geltrex or Matrigel is the typical and most reliable method to keep the pluripotent states of many hiPS and hES cell lines in feeder-free conditions.⁶⁰ However, these culture conditions are not chemically defined and contain xeno-derived molecules. Their xeno-derived molecules hinder the clinical use of hPS cells cultured on Geltrex or Matrigel coating dishes.^{61,62}

It is critical to develop cell culture biomaterials that support large-scale production of hES cell and hiPS cell lines under xeno-free and feeder-free systems, which are compliant with cGMP (current good manufacturing practice).^{63–70} The use of feeder layers restricts the use of hPS cells in clinics. Studies have reported in detail several alternative hPS cell cultivation methods, which have no feeder layers.

Recently, several cell cultivation matrices were reported to cultivate hPS cells that support their pluripotency in chemically defined media. The recently developed biomaterials need a combination of specific cultivation media, and specific hPS cells may specifically support their pluripotent state on the cell cultivation biomaterials.⁷¹ Moreover, it is currently difficult to choose the ideal and best biomaterials for hPS cell cultivation, although rVN and LN-521 are starting to be used as a gold standard of cell culture matrices for hPS cell culture.

Chapter 3 discusses in detail the current developments in hPS cell cultivation biomaterials and discusses the material-assisted regulation of hPS cells under xeno-free and feeder-free and cell cultivation conditions.

Some strategies can be considered for development of materials for hPS cell cultivation under chemically defined, xeno-free and feeder-free systems. The strategies are hPS cell cultivation (1) on two-dimensional (2D) biomaterial immobilized natural extracellular matrices (ECMs), (2) on or within 2D or 3D hydrogels made from polysaccharide such as GAG (glycosaminoglycan), (3) on 2D biomaterial immobilized synthetic oligopeptides derived from ECMs, (4) on 2D plates made from synthetic polymers, (5) in porous or hydrophilic 3D microcapsules, and (6) on 3D microfibers with or without ECM immobilization.

1.5 hPSC Differentiation on Biomaterials

It is evident that people frequently experience the loss or damage of tissues or organs as a result of birth defects, accidents or disease.^{60,72} Regenerative medicine and tissue engineering may be greatly aided by the use of stem cells, such as hES cells⁴² and hiPS cells.^{26,40,73} The use of synthetic and natural polymer materials to mimic stem cell microenvironments and niches can help generation of significant numbers of stem cells and help produce the widely differentiated cells necessary for *in vitro* translational medicine. Hormones, ECM, and small chemical molecules are biological cues that determine the pluripotency of stem cells and their differentiation fates.^{38,74–78} However, researchers have only recently started to consider how external forces (*e.g.*, light signals, magnetic forces, and electrical forces),^{79,80} mechanical force (*e.g.*, shear stress imposed by cultivation medium and cyclic stretching of biomaterials),⁸¹ polymeric biomaterial stiffness,⁸² cell culture, cell shape, and other such physical cues impact on the induction of stem cells. The protocols and differentiation methods employed with hiPS and hES cells are significantly different from those employed with adult stem cells because of the much higher pluripotent state of hPS cells.

Furthermore, hiPS and hES cells cannot generally be cultivated in conventional polystyrene cultivation plates,^{60,72} because these induce random (spontaneous) differentiation of hiPS and hES cells. On the other hand, adult stem cells can expand and differentiate in polystyrene plates and they can be controlled by the induction medium.

Therefore, Chapter 4 discusses the physical cues of synthetic and natural polymeric materials that lead to the differentiation of hES cells and hiPS cells into several different lineages. Such lineages include dopamine-secreting neurons, neural cells, insulin-secreting beta cells, hepatocytes, osteoblasts, chondrocytes, and cardiomyocytes. In this book, the physical cues of materials that we focus upon are (1) the elasticity of polymeric biomaterials, (2) the topography of polymeric biomaterials, and (3) the mechanical forces associated with materials (electrical stimulation *via* materials and stretching of materials) used for hPS cell cultivation.

1.6 Biomaterials Control hPS Cell Differentiation Fate

Pluripotent stem cells generated from iPS and ES cells have the potential to induce into several cell types, which are derived from the three germ layers: ectoderm cells (epidermal cell, retinal pigment epithelium, dendrocyte, astrocyte, and neuron), mesoderm cells (blood cell, cardiomyocyte, chondrocyte, and osteoblast), and endoderm cells (lung cell, hepatocyte, and β cells, hepatocyte). However, it is challenging to control the induction of pluripotent stem cells, especially hPS cells, into targeted cell lineages because of their variety of induction ability of differentiation.

The stem cell induction is regulated by some independent factors in the hPS cell microenvironment: (1) cell–material interactions in cell cultivation; (2) physical factors, such as cell shape and size, oxygen concentration, shear stress, and the stiffness of the cell cultivation materials; (3) cell–cell interactions, such as in co-cultivation; and (4) bioactive molecules, such as vitamins, cytokines, growth factors, and small molecules.⁶⁰ An excellent strategy is to mimic the stem cell microenvironment for the induction of hPS cells into desired cell lineages using appropriate materials for hPS cell cultivation. The protocol for induction of hPS cells is more complicated because of the high differentiation potential and high pluripotency of hPS cells as well as different cultivation protocols for hPS cells in comparison to human adult stem cells, although human adult stem cells, such as amniotic fluid stem cells, adipose-derived stem cells, dental pulp stem cells, and bone marrow-derived stem cells can be differentiated using simple protocols, such as stem cell culture on materials in differentiation media. Typically, hPS cells are cultivated (1) on Matrigel coating dishes, (2) on feeder cells, such as MEF (mouse embryonic fibroblasts, feeder layer), or (3) on appropriate materials to keep their pluripotency;^{60,72} while human adult stem cells can be cultivated on typical TCP (tissue culture polystyrene) plates. Subsequently, the

hPS cell induction method is extensively different from the method for human adult stem cells. Chapter 5 describes several protocols for inducing hPS cells cultivated on materials and considers the appropriate materials for hPS cell induction into targeted cell lineages.

1.7 Stem Cell Therapy Using Biomaterials

The hPS cells are valuable cell sources to cure injured organs or tissues because of their potential to induce differentiation into many cells derived from the three embryonic germ layers in the human body.

At present, clinical trials of stem cell therapy using hPS cells have only been reported for four cases according to the ClinicalTrials.gov database. These cases are (1) macular degeneration (namely Stargardt macular dystrophy and age-related macular degeneration), (2) acute myocardial infarction (AMI), (3) diabetes, and (4) spinal cord injury. Recently, hPS cell-based therapy in clinical trials has been studied.^{37,83–86} We discuss the current situation of stem cell therapy using hPS cells for patients with (1) myocardial infarction (MI) and (2) macular degeneration, considering the bioengineering points of the therapy in Chapter 6. Moreover, we consider clinical trials using adult or human fetal stem cells such as human mesenchymal stem (hMS) cells that are prepared to cure patients with these diseases.

The goal of Chapter 6 is (1) to describe the use of materials in clinical and preclinical works of hPS cells, hMS cells, and human fetal stem cells; (2) to describe the gap between clinical trials and fundamental research using human stem cells; (3) to describe the current situation of clinical trials of hPS cell-based therapy in comparison to clinical trials using hMS cells and human fetal stem cells; and (4) to consider the bioengineering trends to facilitate hPS cell, hMS cell, and human fetal stem cell therapy in translational medicine.

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CHAPTER 2

Adult Stem Cell Culture on Extracellular Matrices and Natural Biopolymers

2.1 Introduction

The extracellular matrix (ECM) is the extracellular part of animal tissues, which maintains structural back-up for the stem cells, as well as inspiring many key biological properties.¹ ECM proteins can ascertain whether stem cells will multiply or undergo growth retardation, differentiate or maintain static, and expand or undergo apoptotic death.² Then, the ECM proteins are significant causes in recreating the biological roles of stem cells *in vitro* that help stem cells to induce into various heredities, *e.g.*, β -cells, hepatocytes, neural cells, cardiomyocytes, adipocytes, chondrocytes, and osteoblasts. The differentiation of stem cells in culture relies upon the origin, structure, components, and amount of ECMs that are used. Because ECMs are used as matrices or hydrogels for the arrangement of cells in tissues, ECMs are the major cell cultivation ingredients used to checkmate the differentiation and expansion of stem cells in regenerative medicine and translational medicine, both *in vivo* and *in vitro*. To this end, this section centers primarily on the differentiation of stem cells cultivated on materials composed of appropriate ECMs and on the chemical and biological contact between stem cells and ECMs.¹

2.2 Chemical and Biological Interactions of ECM Proteins and Stem Cells

ECM proteins have chemical functional groups of amine, carboxylic acid, phosphate and sulfonic acid. They additionally have characteristics of

polyelectrolytes and therefore, have an isoelectric point (IEP).^{3–18} Table 2.1 demonstrates the IEP of several ECM proteins, natural biopolymers, and growth factors.^{3–15,17,18} Collagen (COL) type I and gelatin gel have IEPs of 4.7–8.3,^{15,17} alginate 5.4,¹⁸ agarose 5.5,⁹ hyaluronic acid (HA) 2.5,¹³ heparin 4.7,⁶ vitronectin (VN) 4.8–5.3,⁴ laminin (LN) 4.9–5.9,⁵ and fibronectin (FN) 5.5–6.0.³ Typical natural biopolymers and ECM proteins are negatively charged under physiological states. The IEP of several growth factors is less than 7 (*e.g.*, 5.3 for insulin¹¹ and 5.6 for FGF-1¹²), while for other growth factors, it is greater than 7 (*e.g.*, 9.5 for TGF- β 1,⁷ 9.8 for PDGF,⁸ 9.0 for BMP-2,¹⁴ and 9.6 for FGF-2¹²). The bonding between growth factors and ECM proteins (*e.g.*, BMP-2 and collagen type I) is mainly as a result of electrochemical interactions.

The binding of ECMs to cells is contemplated by integrin receptors. Integrins are a large group of cell-surface receptors, which mediate and attach to ECMs, arrange the cytoskeleton, and cause activation of intracellular signaling pathways.¹ Each integrin comprises of two kinds of transmembrane subunits: β and α . Eight β and 18 α subunits associate in different

Table 2.1 Isoelectric point (IEP) of some ECM proteins, growth factors, and polymers.¹ Reproduced from ref. 1 with permission from the American Chemical Society, Copyright 2012.

Materials	Isoelectric point
ECM	
Collagen type I	4.7, 6.4, 6.78, 7.02, and 8.26 depending on preparation conditions
Gelatin sol	7.8 temp >40, or increasing pH
Gelatin gel	4.7 temp <15 or decreasing pH
Fibronectin	5.5–6.0
Vitronectin	4.75–5.25
Laminin	5.87, 4.89, and 5.08
Heparin	3.4
Hyaluronic acid	2.5
Growth factor	
FGF-1 (aFGF)	5.6
FGF-2 (bFGF)	9.6
rhBMP-2	9
Insulin	5.3
PDGF	9.8
EGF	4.0–5.0
TGF- β 1	9.5
Polymer	
Agarose	5.5
Alginate	5.4
Poly(lactic-co-glycolic acid) (PLGA)	2.75
Poly(L-lysine)	9.5
Chitosan	8.7
Polyacrylamide	5.7

partnerships to generate 24 integrin dimers in mammals, which are able to bind to specific subsets of ECM ligands.^{19,20}

Typical ECMs have molecular weights of 10–1000 kDa, which have only small numbers of integrin-binding sites. These integrin-binding sites have definite arrangements of a few amino acids (3–10), *e.g.*, GFOGER, IKVAV, YIGSR, DGEA, and RGD. Table 2.2 shows the amino acid sequences and integrin receptors that can mediate ECM–cell associations. The ECM–cell associations are a key factor for MS (mesenchymal stem) cell and other stem cell differentiation and expansion, as well as for typical cell cultivation.

Table 2.2 ECM-mimicking peptides immobilized on dishes for adhesion, differentiation, and proliferation of stem cells.¹ Reproduced from ref. 1 with permission from the American Chemical Society, Copyright 2012.

ECM-mimicking peptide	ECM for mimicking	Binding site of cells
DGEA	Collagen I	Integrin ($\alpha_2\beta_1$)
GTPGPQGIAGQRGVV (P15)	Collagen I	Integrin ($\alpha_2\beta_1$)
(RADA)4GGDGEA	Collagen I	Integrin ($\alpha_2\beta_1$)
(RADA)4GGFPGERGVEGPGP	Collagen I	
GFOGER	Collagen	Integrin ($\alpha_2\beta_1$)
MNYYSNS	Collagen IV	
RGD	Collagen I	Integrin ($\alpha_v\beta_3$)
ELIDVPST (CS-1)	Fibronectin	Integrin ($\alpha_4\beta_1$); VLA-4
FN-40	Fibronectin	Integrin ($\alpha_4\beta_1$); VLA-4
FN-120	Fibronectin	Integrin ($\alpha_5\beta_1$); VLA-5
FN-CH296	Fibronectin	Integrin ($\alpha_4\beta_1$, $\alpha_5\beta_1$)
KGGAVTGRGDSPASS	Fibronectin	Integrin ($\alpha_5\beta_1$); VLA-5
GRGDSPK	Fibronectin	Integrin ($\alpha_5\beta_1$); VLA-5
KNNQKSEPLIGRKKT	Fibronectin	Heparin-binding domain
RGDS	Fibronectin	
PHSRN	Fibronectin	
KYGAASIKVAVSADR	Laminin	
YIGSR	Laminin	
IKVAV	Laminin	
PPFLMLLKGSTR	Laminin-5 (Laminin332)	Integrin ($\alpha_3\beta_1$)
NPWHSIYITRFG (AG10)	Laminin α_1	Integrin ($\alpha_6\beta_1$)
AGQWHRVSVRWG (A5G81)	Laminin α_5	Integrin ($\alpha_6\beta_1$)
GTTSWSQCSKS (T1)	Laminin	Integrin ($\alpha_6\beta_1$)
KIKMVISWKG (HYD1)		Integrin ($\alpha_6\beta_1$)
VSWFSRHRYSPPFAVS (P3)		Integrin ($\alpha_6\beta_1$)
CGLPYSSVC (N4)		Integrin ($\alpha_6\beta_1$)
(RADA)4-GG PDSGR	Laminin	
(RADA)4-GGSDPG YIGSR	Laminin	
(RADA)4-GGIKVAV	Laminin	
KGGPQVTRGDVFTMP	Vitronectin	Integrin ($\alpha_v\beta_5$)
KGGNGEPRGDTYRAY	Bone sialoprotein (BSP)	
PEO4-NGEPRGDTYRAY	BSP-linker	
RGD (HAVDI)	Osteopontin (N-cadherin)	Integrin ($\alpha_v\beta_3$) (N-cadherin)

Most of the integrin family, including $\alpha_V\beta_8$, $\alpha_V\beta_6$, $\alpha_V\beta_5$, $\alpha_V\beta_3$, $\alpha_{IIb}\beta_3$, $\alpha_8\beta_1$, and $\alpha_5\beta_1$ use an RGD (Arg–Gly–Asp) domain within von Willebrand factor, vitronectin,²¹ fibrinogen,²² FN,^{21–23} and other glycoproteins. COL type I holds the cell-binding site of DGEA that interacts with integrin $\alpha_2\beta_1$.²⁴ COL type I can bind to integrin $\alpha_V\beta_3$, $\alpha_3\beta_1$, and $\alpha_1\beta_1$.^{25,26} RGD on COL type I is known to interact with integrin $\alpha_V\beta_3$.²⁵ The gigantic size of ECMs in comparison to the integrin-binding sites of small size, gives morphological assistance as well as configurational control of the cell attaching sites. The distinctions in configuration of the cell attaching sites cause distinct interactions with specific integrin receptors.^{27,28} MS cell differentiation on cultivation biomaterials comprised of several natural biopolymers and ECM proteins is debated in the following sections.

2.3 Collagen

COL is a common protein of ECMs used in the cultivation of MS cells that is distributed in animals, particularly in the connective tissue and flesh of humans.²⁹ COL is a major molecule of connective tissues and the most abundant protein in humans,³⁰ accounting for approximately 25–35% of the body's entire protein composition. Extended COL fibrils are observed in fibrous tissue such as tendons, ligaments, and skin. COL is also found in large amounts in the intervertebral discs, gut, blood vessels, bone, cartilage, and cornea. Due to its abundance, COL (particularly COL type I) is inexpensive compared to other ECM proteins such as FN, vitronectin, and LN. This permits us to use COL type I in large amounts to generate hydrogels or scaffolds for stem cell cultivation.^{31–35}

Twenty-nine COL have been noted and reported to date. Five typical COLs are: (1) COL type V (genes; COL5A3, COL5A2, and COL5A1), which is included in hair and placenta;³⁶ (2) COL type IV (genes; COL4A6, COL4A5, COL4A4, COL4A3, COL4A2, and COL4A1) that is seen in basement membranes;³⁷ (3) COL type III (gene; COL3A), which is the main component of reticular fibers; (4) COL type II (gene; COL2A), which is the main component of cartilage; and (5) COL type I (genes; COL1A2 and COL1A1), which is the main component of bone and is also found in skin and tendons.

COL passes through several post-translational modifications, containing significant crosslinking. Defective crosslinking has been observed in human syndromes (e.g., Ehlers–Danlos syndrome and osteogenesis imperfecta).³⁸ Conversely, it was found that the restriction of crosslinking of COL was not needed for osteogenic differentiation of human MS (hMS) cells, as described by the expression of alkaline phosphatase (ALP) and genome-wide gene expression assay, although the crosslinking of COL did increase matrix mineralization.³⁸ Particular features of COL, such as degree of crosslinking, elasticity, stiffness, and origin (*i.e.*, fish, pig, or cow originated COL from adult or fetal animals) could influence stem cell fate when COL is used in the cultivation scaffolds and biomaterials for MS cell induction.

COL is able to generate scaffolds or hydrogels with no elaboration. COL is dissolved in acetic acid solution to prepare COL hydrogels, and the solution is mixed with buffer solution. When fitting the pH of COL solution at 7.4 with the titration of sodium hydroxide solution, the COL solution is cooled in cold water to inhibit solidification. Cells are subsequently put together in the COL solution at the required concentration, and the cell solution is cultured at 37 °C to produce the hydrogel state. When the hydrogels have been generated, extra cultivation media are inputted into the apex of the hydrogels and the hydrogels containing cells are incubated in the incubator.³⁹ Tables 2.3 and 2.4 show some types of COL biomaterials and matrices (scaffolds) for MS cell induction, which have been described by several researchers.^{25,31–35,39–111}

2.3.1 Collagen Type I Scaffold

COL scaffolds (sponges) are prepared by the traditional freeze-drying protocol accompanied by crosslinking.^{31,77} COL type I is typically used for matrices and cultivation biomaterials to facilitate osteogenic^{32,33,57,65,68,112} and chondrogenic³³ induction of MS cells.

Several studies have centered on the osteogenic differentiation of MS cells cultivated on COL type I matrices,^{33,57} because COL type I is a major organic component of bone.⁵⁴ Stimulation of required integrins ($\alpha_2\beta_1$ and $\alpha_1\beta_1$) with COL type I was found to activate the osteogenic response of hBMS (human bone marrow stem) cells.^{25,38,49,57,69}

The expansion and osteogenic induction of MS cells on collagen type I-coated scaffolds and dishes are valuable. Studies have found that the tissue culture polystyrene (TCP) dishes immobilized with COL type I, but not poly-L-lysine (PLL), gelatin, LN or FN promoted late cell proliferation and guided hBMS cell osteogenesis, as exhibited by an enhancement in ALP activity, Alizarin Red S staining, and mRNA intensity of Runx2 (early marker of osteogenesis) and osteocalcin.⁶⁷ Tsai and his colleagues reported that a COL type I coated surface generated the stimulation of ERK (extracellular signal regulated kinase) as well as Akt (serine/threonine protein kinase), and not FAK (focal adhesion kinase).⁶⁷ The inhibition of $\alpha_2\beta_1$ integrin by the antibody did not prevent COL type I-induced osteogenic differentiation of hBMS cells.⁶⁷ These results indicate that cell signaling through $\alpha_2\beta_1$ integrin is not necessary for osteogenic differentiation of hBMS cells cultivated on COL type I.

Donzelli and his colleagues investigated rat MS cell osteogenesis on Gingistat[®] (commercial COL scaffolds). MS cell commitment to osteogenic induction was evaluated by the expression of osteocalcin and osteopontin, and enhanced activity of ALP. Alizarin Red-stained calcium deposits and nodular aggregates were found in MS cells induced towards osteogenic differentiation cultivated in the COL matrices.³³

A honeycomb morphology of COL matrices was found to facilitate BMS cell differentiation and proliferation.⁵⁸ BMS cells on honeycomb COL

Table 2.3 Investigations into stem cell induction on 2D COL biomaterials.¹ Reproduced from ref. 1 with permission from the American Chemical Society, Copyright 2012.

Stem cell source ^a	Material for stem cell culture	Differentiation	Ref.
hBMSCs	Collagen I (2D culture, coating on dishes)	Osteoblasts	25, 62, 65, 66, 67, 110
Rat BMSCs	Collagen I (2D culture, coating on dishes)	Osteoblasts	68
Rat BMSCs	Collagen I/BMP-2 (2D culture, coating on dishes)	Osteoblasts	93
rBMSCs	Collagen I (2D culture, gel)	Osteoblasts	69
hADSCs	Collagen I (2D culture, PEM)	Osteoblasts	108
mBMSCs	Collagen I (2D culture, coating on dishes)	Osteoblasts, adipocytes	70
hBMSCs	Collagen I (2D culture, coating on dishes)	Osteoblasts, adipocytes	71
hBMSCs	Collagen I (2D culture, aligned collagen on dishes)	Osteoblasts, adipocytes	60
hBMSCs	Collagen I (2D culture, aligned heparin on collagen I matrix)	Osteoblasts, adipocytes	60
pBMSCs	Collagen I (2D culture, coating on dishes)	Osteoblasts, adipocytes	72
hBMSCs	Collagen I (2D culture, coating on dishes)	Osteoblasts, chondrocytes	47
hADSCs	Collagen I (2D culture, coating on dishes)	Adipocytes	45
hBMSCs	Collagen I (2D culture, coating on dishes)	Vascular smooth muscle cells	59
hESCs (TE03, TE06)	Collagen I (2D culture, coating on dishes)	Neural cells	51
hBMSCs	Collagen I (2D culture, coating on dishes)	Neural cells	56, 64
mESCs	Collagen I (2D culture, coating on dishes)	Neural cells	73
mESCs	Collagen I (2D culture, coating on dishes)	Lung epithelium	76
Monkey ESCs	Collagen I (2D culture, coating on dishes)	Mesoderm cells, endoderm cells	74
Mouse hepatic stem cells	Collagen I (2D culture, coating on dishes)	Hepatocytes	75
hBMSCs, hAFSCs	Collagen I (2D culture, coating on dishes)	Hepatocytes	44
Human neural stem cells	Collagen I (2D culture, coating on dishes)	Oligogliocytes	41
Teratocarcinoma stem cells (F9)	Collagen I (2D culture, coating on dishes)	Visceral endoderm cells	55
Rat BMSCs	Collagen I (2D culture, nano-sized collagen addition)	Insulin-secreting cells	109
hBMSCs	Collagen IV (2D culture, coating on dishes)	Osteoblasts	25
hADSCs	Collagen IV (2D culture, coating on dishes)	Adipocytes	45

Table 2.3 (Continued)

Stem cell source ^a	Material for stem cell culture	Differentiation	Ref.
hBMSCs	Collagen IV (2D culture, coating on dishes)	Neural cells	56
Mouse hepatic stem cells	Collagen IV (2D culture, coating on dishes)	Hepatocytes	75
Teratocarcinoma stem cells (F9)	Collagen IV (2D culture, coating on dishes)	Visceral endoderm cells	55
hBMSCs	Collagen IV (2D culture, coating on dishes)	Smooth muscle cells	75

^aADSC, adipose-derived stem cells; BMSCs, bone marrow stem cells; ESCs, embryonic stem cells; hADSCs, human ADSCs; hAFSCs, human amniotic fluid-derived stem cells; hBMSCs, human BMSCs; hESCs, human ESCs; mBMSCs, mice BMSCs; mESCs, mice ESCs; pBMSCs, porcine BMSCs; PEM, polyelectrolyte multilayer; rBMSCs, rabbit BMSCs.

scaffolds could induce differentiation into osteoblasts even with no osteogenic differentiation supplement, as described by ALP activity and consideration of mineral deposition evaluated from von Kossa staining assay.⁵⁸

COL type I nanofibrous scaffolds were made by an electrospun method and inoculated with hBMS cells.³² The cell motility, adhesion, growth, morphology, and osteogenic induction of hBMS cells on nano-sized fibers of different diameters (500–1000, 200–500 and 50–200 nm) were investigated. The cells on any nanofibrous scaffolds had more flattened and polygonal cell shapes than those on TCP plates. Furthermore, hBMS cells cultured on 500–1000 nm nanofibers showed much higher cell viability than the cells on TCP dishes.³²

Sefcik and his colleagues also made COL type I nanofibrous matrices by the electrospun system.³⁴ Osteogenic genes (Runx2, COL type I, ALP, osteocalcin, osteopontin, and osteonectin) were found to be elevated (>1-fold) in adipose-derived stem (ADS) cells cultivated on nanofibrous matrices in comparison to two-dimensional (2D) COL coating surface until 3 weeks of culture.³⁴ High synthesis of mineralized ECM was found on the nanofibrous matrices evaluated from Alizarin Red staining evaluation on 3 weeks of culture. The results indicate that three-dimensional (3D) nanoscale morphologies play an important function in controlling cell fate determination and *in vitro* osteogenesis of ADS cells in serum-free culture conditions.³⁴

Chondrogenesis of MS cells promoted by COL type I hydrogels was studied by several researchers.^{48,85,86,113} Chang and his colleagues investigated the comparison of chondrogenesis of immortalized hBMS cells entrapped in COL type I hydrogels with the cells grown in pellet cultivation.¹¹³ The hBMS cells in COL hydrogels showed more glycosaminoglycan than those in pellet cultivation (a gold standard method of chondrogenesis of MS cells). Expression of the chondrogenic genes COL type II, aggrecan, Sox9, and COL type I (which results in de-differentiation) enhanced over time in pellet cultivation. However, Sox9 expression was not changed and COL type I

Table 2.4 Investigations into stem cell induction on 3D COL biomaterials.¹ Reproduced from ref. 1 with permission from the American Chemical Society, Copyright 2012.

Stem cell source ^a	Material for stem cell culture	Differentiation	Ref.
rBMSCs	Collagen I (3D culture, gel)	Osteoblasts	68
Rat BMSCs	Collagen I (3D culture, gel)	Osteoblasts	57
hBMSCs	Collagen I (3D culture, scaffold)	Osteoblasts	77
rBMSCs	Collagen I (3D culture, scaffold)	Osteoblasts	33
hBMSCs	Collagen I (3D culture, crosslinked scaffold)	Osteoblasts	35, 65
hADSCs	Collagen I (3D culture, electrospinning nanofiber)	Osteoblasts	34
rADSCs	Collagen I/PBLG/HYA (3D culture, electrospinning nanofiber)	Osteoblasts	94
hBMSCs	Collagen I (3D culture, electrospinning nanofiber)	Osteoblasts	32, 78
rBMSCs	Collagen I/PGA fiber (3D culture, sponge)	Osteoblasts	31
Rat BMSCs	Collagen I/bioglass/PSN (3D culture, scaffold)	Osteoblasts	79
rBMSCs	Collagen I/PGA (3D culture, sponge)	Osteoblasts	80
hBMSCs	Collagen I/HYA (3D culture, scaffold)	Osteoblasts	66
hBMSCs	Collagen/PVP-Iodide (3D culture, scaffold)	Osteoblasts	95
rBMSCs	Collagen I/chitosan (3D culture, sponge)	Osteoblasts	81
hBMSCs, Wharton's jelly of UCB	Collagen I/collagen III (3D culture, scaffold)	Osteoblasts	40
rBMSCs	Collagen I/chondroitin-6-sulfate (3D culture, scaffold)	Osteoblasts, chondrocytes	46
hBMSCs	Collagen I/HYA (3D culture, scaffold)	Osteoblasts, chondrocytes	49
hBMSCs	Collagen/HYA (3D culture, scaffold)	Osteoblasts, chondrocytes	96
pBMSCs	Collagen I/PCL/TCP (3D culture, scaffold)	Osteoblasts, adipocytes	72
hBMSCs, hUCB-MSCs	Collagen I/collagen III (3D culture, gel)	Osteoblasts, adipocytes	40
bBMSCs	Collagen I (3D culture, gel)	Chondrocytes	61
hBMSCs	Collagen I (3D culture, gel)	Chondrocytes	48
hADSCs	Collagen I (3D culture, gel)	Chondrocytes	50
mESCs	Collagen I (3D culture, gel)	Chondrocytes	82
hBMSCs	Collagen I (3D culture, sponge)	Chondrocytes	83
rBMSCs	Collagen I (3D culture, microsphere)	Osteochondrocytes	84
hBMSCs	Collagen I (3D culture, microsphere)	Chondrocytes	85
rBMSCs	Collagen (3D culture, microsphere)	Chondrocytes	97
bBMSCs	Collagen I/alginate (3D culture, gel)	Chondrocytes	54
rBMSCs	Collagen I/alginate (3D culture, gel)	Chondrocytes	86

Table 2.4 (Continued)

Stem cell source ^a	Material for stem cell culture	Differentiation	Ref.
mBMSCs	Collagen/silk fibroin (3D culture, electrospinning nanofibers)	Chondrocytes	98
hBMSCs	Collagen I/HA/PCL (3D culture, scaffold)	Chondrocytes	63
tMSCs	Collagen/HA (3D culture, gel)	Chondrocytes	111
Rat cardiac stem cells	Collagen I/PLGA (3D culture, scaffold)	Cardiomyocytes	87
mESCs	Collagen I/Matrigel (3D culture, scaffold)	Cardiomyocytes	88
mBMSCs	Collagen I immobilized Sca-1 antibody (3D culture, scaffold)	Cardiomyocytes	42
hADSCs	Collagen (3D culture, scaffold)	Tendon	99
Rat ADSCs	Collagen/nanoparticle (3D culture, fibers)	Tendon	100
hBMSCs	Collagen I/PLCL (3D, electrospinning nanofiber)	Neural cells	43
Rat BMSCs	Collagen/CNT (3D culture, gel)	Neural cells	101
NSCs	Collagen I (3D culture, grafting on electrospinning mat)	Neural cells	89
NSCs	Collagen I (3D culture, gel)	Neural cells	39
Rat NSCs	Collagen I (3D culture, gel)	Neural cells	92
Rat NSCs	Cetuximab modified collagen (3D culture, scaffold)	Neural cells	102
Rat NSCs	Heparinized collagen (3D culture, conduit)	Neural cells	103
Mice NSCs	Collagen I (3D culture, gel)	Neural cells	90
Mice NSCs	Collagen I/laminin (3D culture, gel), collagen I/fibronectin (3D culture, gel)	Neural cells	90
Rat stem cells	Collagen I (3D culture, gel)	Neuronal circuits	91
hBMSCs	Fibroblast-embedded collagen I (3D culture gel)	Epidermis	53
hAFSCs	Collagen/chondroitin sulfate (3D, scaffold)	Endothelial cells	104
hBMSCs	Collagen/PLA-co-PCL (3D culture, electrospinning nanofibers)	Hepatospheres	105
hADSCs	Collagen II (3D culture, gel)	Chondrocytes	50
bBMSCs	Collagen II/alginate (3D culture, gel)	Chondrocytes	54
BMSCs	Collagen II/methacrylamide (3D culture, gel)	Chondrocytes	106
Rat ADSCs	Collagen II/chondroitin sulfate (3D culture, gel)	Nucleus pulposus cells	107
Rat BMSCs	Atelocollagen (3D culture, honeycomb structure)	Osteoblasts	58

^aADSCs, adipose-derived stem cells; bBMSCs, bovine BMSCs; BMSCs, bone marrow stem cells; ESCs, embryonic stem cells; gBMSCs, goat BMSCs; HA, hyaluronic acid; hADSCs, human ADSCs; hBMSCs, human BMSCs; HYA, hydroxyapatite; mBMSCs, murine BMSCs; mESCs, murine ESCs; PBLG, polybenzyl-L-glutamate; pBMSCs, pig BMSCs; PCL, polycaprolactone; PEG, polyethylene glycol; PGA, poly(glycolic acid); PLA-co-PCL, poly(L-lactic acid)-co-poly-(3-caprolactone); PSN, phosphatidylserine; PVP, polyvinylpyrrolidone; rADSCs, rabbit ADSCs; rBMSCs, rabbit BMSCs; tMSCs, tonsil-derived mesenchymal stem cells; UCB, umbilical cord blood.

expression reduced, which suggested that there was no de-differentiation from the chondrogenic lineage, whereas only expression of aggrecan and COL type II in hBMS cells in the COL hydrogels enhanced over time. These results suggest that chondrocytes induced from hBMS cells in COL hydrogels are preferable to the cells produced in pellet cultivation due to their lower amounts of de-differentiation.

The control of embryonic stem (ES) cells in specific lineages of induction is a technically difficult and complicated topic. COL type I microspheres entrapped with mouse ES (mES) cells were described as a preferable environment for maintaining mES cells and supporting their pluripotent conditions for a specific time.⁸² However, Yeung and his colleagues found that the ratio of pluripotent mES cells in the microspheres slowly reduced and the ratio of MS cells was enhanced at later time points.⁸² This result suggests inductive characteristics of the COL matrices for differentiation of mES cells towards MS cell lineages. A lower initial COL concentration promoted mES cell induction into chondrogenic lineages, whereas mES cells induced differentiation into more advanced stages of chondrocytes at a later time point using chondrogenic induction media.⁸² The culture of hES cells and hiPS cells in scaffolds and hydrogels of COL type I or natural biopolymers and other ECMs would generate efficient differentiation into MS cell lineages, which include cardiomyocytes, chondrocytes, and osteoblasts. This idea would support a larger-scale cell production of MS cell lineages that is restricted to autologous patients at present.

Bioengineering complicated tissue that is composed of several tissue domains with various functions and structures is extremely taxing. In particular, it is not easy to generate biological interfaces between mechanically different tissues such as bone and cartilage. The creation of the osteochondral interface with adequate zonal organization is very complicated, and great energy has been put into the development of osteochondral plugs.^{84,114,115} Osteochondral interfaces are important for avoiding mechanical failure and retaining the usual roles of cartilage.⁸⁴

Cheng and his colleagues examined *in vitro* preparation of a stem cell-derived osteochondral interface, with a calcified cartilage interface, which separates an underlying bone layer and a non-calcified cartilage layer using BMS cells stuck to a COL type I microsphere.⁸⁴ Rabbit BMS cells were enclosed in a COL microsphere made of self-assembled non-fibrous meshworks.⁸⁴ BMS cells in the COL microsphere were demarcated into two groups (Figure 2.1); one group was soaked in chondrogenic induction media to guide induction into chondrogenic cells, while the other group was dipped in osteogenic induction media and induced into osteogenic cells. Many cartilage-like and bone-like microspheres were combined to generate osteogenic and chondrogenic layers, respectively.⁸⁴ Layers of the functional subunits were placed in contact with a central pluripotent BMS cell-COL layer in a three-layer morphology for 3D co-cultures. By 5 weeks, calcified cartilage interfaces were created between the non-calcified cartilage layer and the underlying bone layer. The cells located in the interface site were

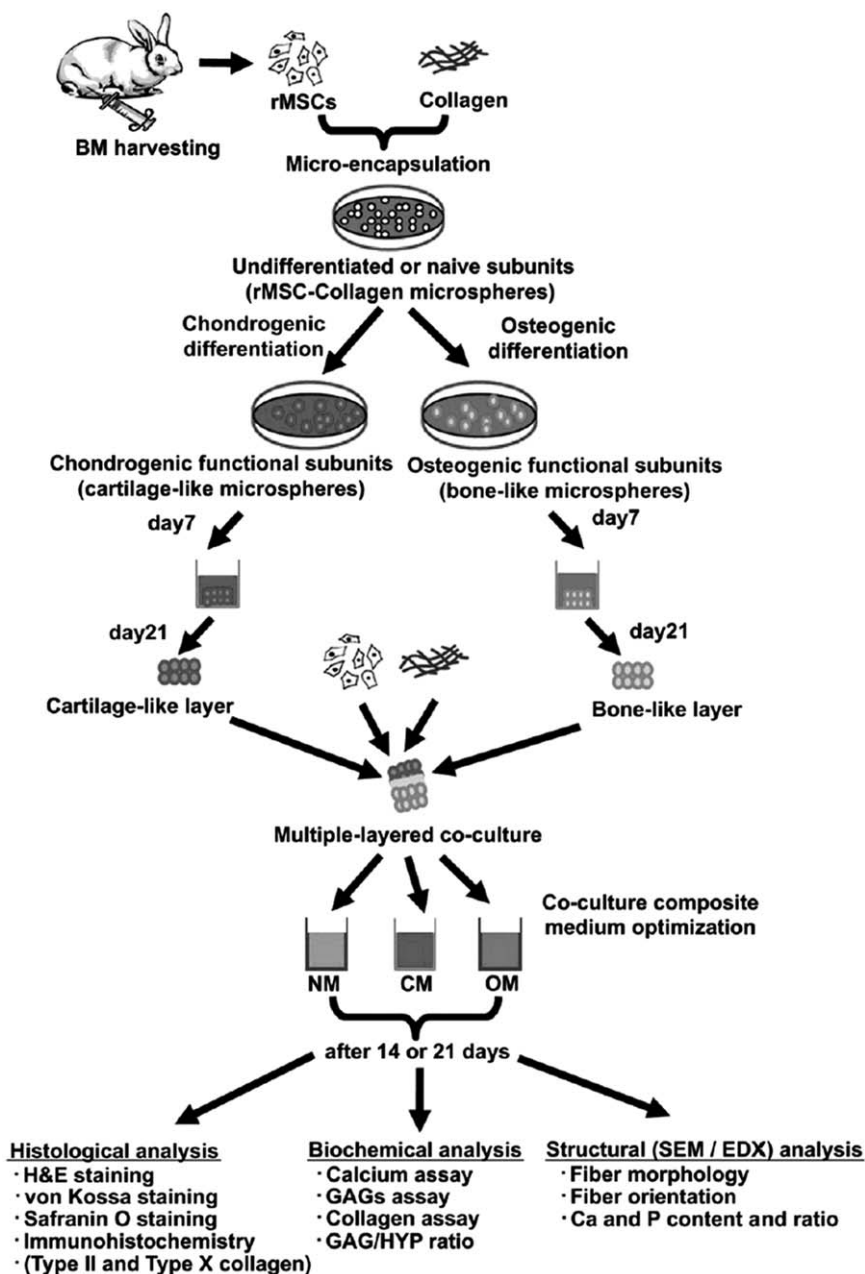


Figure 2.1 Schematic of the overall design in generating the osteochondral interface using rabbit BMS cells (rMSCs) and COL microspheres. NM: Normal medium; CM: Chondrogenic medium; OM: Osteogenic medium.⁸⁴ Reproduced from ref. 84 with permission from Elsevier, Copyright 2011.

observed to be hypertrophic chondrocyte, and ECM in this section consisted of COL type X and II, and calcium deposition. The osteochondral interface was echoed to be widely similar to the native osteochondral interface, based on the existence of glycosaminoglycans (GAGs), COL type II and type X, calcium phosphate deposits, hypertrophic chondrocytes, and vertically aligned COL bundles.⁸⁴ Therefore, an osteochondral construct with appropriate zonal organization can be produced using rabbit BMS cells and COL *in vitro*.

Scaffolds and hydrogels prepared with COL type I have also been examined to facilitate induction of stem cells into neural cells. Ma and his colleagues investigated induction of CNS (central nervous system) mammalian stem cells into neuronal circuits in hydrogels prepared with COL type I.³⁹ The proliferative ability and differentiation potential of neural progenitors in 3D COL hydrogels suggest their potential use to facilitate neuronal regeneration.

2.3.2 Organic Hybrid Scaffold Made of Collagen Type I

The degradation behavior, swelling properties, and mechanical strength of scaffolds, as well as their biocompatibility, ensure crucial roles in the long-term outcome of tissue-engineered stem cell/biomaterial constructs.^{81,116–120} The weak mechanical strength and shrinking of scaffolds are a serious obstacle to the use of purely COL scaffolds in regenerative medicine. Then, natural biopolymers or synthetic polymers are often brought together into COL hydrogels or scaffolds to increase their mechanical strength (Table 2.4). Shrinkage was not found in the hydrogels or scaffolds produced from COL mixed with natural or synthetic polymers inoculated with MS cells. Synthetic polymers, such as poly(glycolic acid) (PGA), poly(lactic-co-glycolic acid) (PLGA), and poly(L-lactic acid)-co-poly(3-caprolactone) (PLCL) as well as natural polymers of HA, chitosan, and alginate are mixed with COL for this object.

It is known that the contractile characteristics of skeletal cells are valuable, and the *in vivo* function of contractility should be explained when designing tissue generation.^{49,121,122} Studies have shown that a decline in contraction induced by changing the crosslinking protocol of glycosaminoglycan–COL matrices led to decreased COL type II secretion by articular chondrocytes.¹²³ Therefore, synthetic biopolymers and malleable ECMs might supply environmental keys, which guide cell fate of induction, and these ideas could be added in scaffold design.

Fujita and his colleagues made three types of scaffolds: a COL type I-PGA (UV) scaffold, a COL type I-PGA scaffold, and a COL type I scaffold inoculated with rat BMS cells.⁸⁰ The COL type I-PGA (UV) scaffold was crosslinked by irradiation with UV light.⁸⁰ The COL type I scaffolds with BMS cells shrank enormously, where COL type I-PGA and COL type I-PGA (UV) scaffolds kept their initial appearance. COL type I-PGA scaffolds without and with crosslinking by UV generated high ALP intensity (indicative of

osteogenic induction) in media including dexamethasone (osteogenic induction molecule). The inclusion of FGF-2 as well as dexamethasone enhanced cell expansion. However, extremely low osteogenic induction of BMS cells was seen in COL type I-PGA, COL type I-PGA (UV) and COL type I scaffolds with no osteogenic supplement in the cultivation media.⁸⁰

Osteoblasts were found to preserve their phenotype and MS cells go to osteogenic differentiation when cultivated in ECMs including COL type I.^{54,124,125} The interplay between $\alpha_2\beta_1$ integrin (major COL type I receptor) and COL type I in MS was responsible for the osteogenesis of MS cells.^{54,125}

Hybrid-type scaffolds made using an easy preparation technique have been examined. The COL type I scaffold can be generated on and in a mechanically tough PLGA knitted mesh. Dai and his colleagues designed three kinds of scaffolds (Figure 2.2): (1) COL sponges made on both sides of the PLGA mesh, (2) COL microsponges made on one side of the PLGA mesh; (3) COL microsponges made in the interstices of the PLGA mesh.¹²⁶ All three groups of implants indicated sufficient cartilaginous ECM deposition, natural chondrocyte morphologies, and uniform cell distribution. The expression of type II COL and aggrecan mRNA and production of glycosaminoglycans were much higher in the COL scaffolds made on both sides or one side of the PLGA mesh than in the COL scaffolds made in the interstices of the PLGA mesh. The engineered cartilage showed 49% (both sides of PLGA mesh) and 55% (one side of PLGA mesh) of the Young's modulus of native articular cartilage and 63% (both sides) and 69% (one side) of the stiffness of the native tissue.¹²⁶ The scaffold could be used for the treatment of articular cartilages with tunable thickness. The platform of the hybrid morphologies provides a possible technique for the production of 3D sponges.

Bishi and his colleagues prepared a biocomposite nanofibrous scaffold composed of poly(L-lactic acid)-co-poly(3-caprolactone)/COL type I (PLA-PCL/COL) to investigate the hepatic differentiation of hBMS cells, because MS cell-based liver tissue engineering on nanofibrous scaffolds may hold promise for cell-based therapies in liver injuries and end-stage liver failure operations.¹⁰⁵

The nanofibrous scaffolds were prepared by an electrospun method and characterized for fiber morphologies, tensile properties, porosity, and

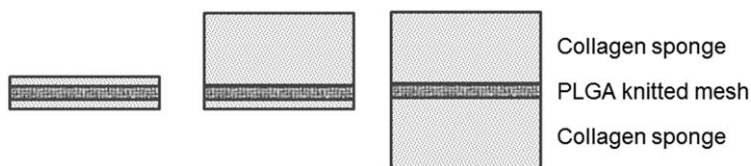


Figure 2.2 Schematic illustration of three structural designs of COL/PLGA hybrid scaffolds. Black: PLGA knitted mesh; Gray: COL type I sponge.¹²⁶ Reproduced from ref. 126 with permission from Elsevier, Copyright 2012.

surface wettability. Hepatic differentiation of hBMS cells was performed on these scaffolds over a period of approximately 1 month using sequential differentiation with hepatogenic growth factors.¹⁰⁵ Hepatic differentiation was evaluated by cell phenotype tracking dye expression, SEM (scanning electron microscopy), albumin release, immunofluorescence staining of hepatocyte-specific markers (AFP, ALB, and CY-18), and quantitative expression of hepatic genes (HNF-4 α , ALB, and AFP).¹⁰⁵ The results suggested that the porous PLA-PCL/COL nanofiber scaffolds supported hBMS cell expansion and hepatic induction in comparison with individual PLA-PCL and COL scaffolds and a monolayer cultivation on TCP dishes.¹⁰⁵ In particular, hBMS cell-derived hepatocyte-like cells on PLA-PCL/COL nanofiber scaffolds could aggregate to generate functional 'hepatospheres', which resemble normal hepatic spheroids (Figure 2.3).¹⁰⁵ This study suggests that the PLA-PCL/COL nanofiber scaffold is extremely biomimetic and upon sequential induction with hepatogenic cytokines/growth factors, which augment induction of hBMS cells towards functional hepatosphere generation.¹⁰⁵ The bioengineered nanofiber scaffolds for hepatic constructs may provide an interesting strategy for cellular therapy of injured livers in end-stage liver failure treatment.

Hybrid scaffolds consisting of natural biopolymers and COL type I have been investigated for treatment of cartilage, bone, and other tissues. Scaffolds consisting of glycosaminoglycan and COL type I have been prepared by

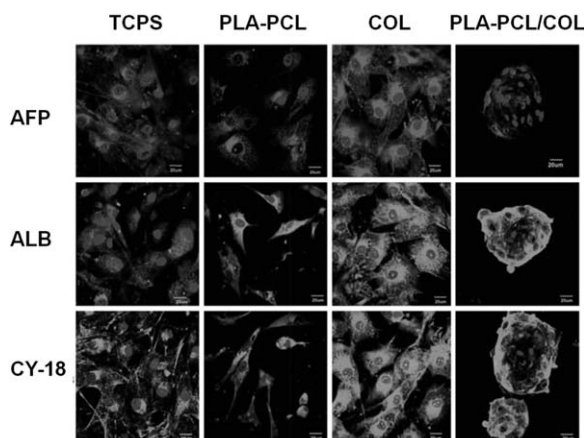


Figure 2.3 Confocal microscopy merged pictures showing expression of hepatocyte-specific markers, AFP (Alexa Fluor 594), ALB (Alexa Fluor 488), and CY-18 (FITC) for hepatocyte-like cells generated on TCP, PLA-PCL, and COL and hepatospheres formed on PLA-PCL/COL nanofiber scaffolds at day 28. Nuclei were stained with DAPI. Three-dimensional hepatospheres on PLA-PCL/COL nanofiber scaffolds exhibited strong expression of albumin (ALB) and cytokeratin-18 (CY-18) and weak expression of α -fetoprotein (AFP) (scale bar = 20 μ m).¹⁰⁵ Adapted from ref. 105 with permission from The Royal Society of Chemistry.

some investigators for regenerative medicine using stem cells.⁴⁶ Farrell and his colleagues made scaffolds consisting of chondroitin-6-sulfate and COL type I. Rat BMS cells underwent osteogenesis when the cells were cultivated on the scaffold and activated with osteogenic factors (β -glycerophosphate, ascorbic acid, and dexamethasone), as examined by expression of osteocalcin and COL type I as well as mineral deposition evaluated by von Kossa and Alizarin Red staining.⁴⁶ The stimulation by osteogenic factors was associated with activation of ERK, which plays an essential part in osteogenic differentiation of MS cells.⁴⁶

Chitosan is a partially deacetylated polymer of chitin, which is suitable for osteoblast culture.^{81,127} Arpornmaeklong and his colleagues formed hybrid sponges (scaffolds) consisting of chitosan-COL type I for osteogenic induction of rat BMS cells to improve the biological and mechanical characteristics of COL scaffolds.⁸¹ The BMS cells adhered extensively to the scaffolds. The expression of osteocalcin and ALP on chitosan-COL type I and COL composite scaffolds were higher than that on chitosan scaffolds. A 1:1 COL-chitosan scaffold exhibited the highest compression strength.⁸¹ Therefore, a combination of COL-chitosan matrices facilitated osteoblastic induction of BMS cells and showed an improvement in their physical and mechanical characteristics.

Ma and his colleagues prepared rat-tail COL-based nerve conduits for repair of lengthy facial nerve defects, which promote neural progenitor/stem (NPS) cell expansion in the natural nerve conduit with anchored bFGF (FGF-2) to promote the therapeutic effect of cell implantation.¹⁰³ Introducing NPS cells for repairing facial nerve injuries could be a strategy for nerve gap regeneration. However, the lack of success using the present protocols of applying NPS cells to neurological disease is due to poor engraftment followed by implantation into the host tissue, which may be improved by using the rat-tail COL-based nerve conduit anchored bFGF developed in this study.¹⁰³

bFGF is known to bind strongly to exogenous heparin sulfate (HS) with no loss of its bioactivity.¹²⁸ Therefore, heparin was crosslinked to rat-tail COL-derived natural nerve conduit (HS-bFGF-COL) for the controlled release of bFGF to facilitate NPS cell proliferation in this study (Figure 2.4).¹⁰³ The efficacy of HS-bFGF-COL nerve conduit was evaluated in rat models of facial nerve injury.

In vitro investigation described that heparinized COL restricted leakage of bFGF and NPS cells proliferated in the rat-tail COL hydrogels with the anchored bFGF.¹⁰³ The nerve conduits were transplanted to connect 8 mm facial nerve defects in rats. The repair outcomes such as remyelination analysis, immunohistochemistry, electrophysiological tests, and vibrissae movements of regenerated nerve were examined. At 3 months after implantation, only the HS-bFGF-COL nerve conduit treated group exhibited survival of NPS cells. Also, the HS-bFGF-COL nerve conduit with NPS cells greatly facilitated nerve growth and functional recovery that was similar to those of an autograft case, the current gold standard method.¹⁰³ The animal experimental results using the HS-bFGF-COL nerve conduit with NPS cells were important for facial nerve regeneration.

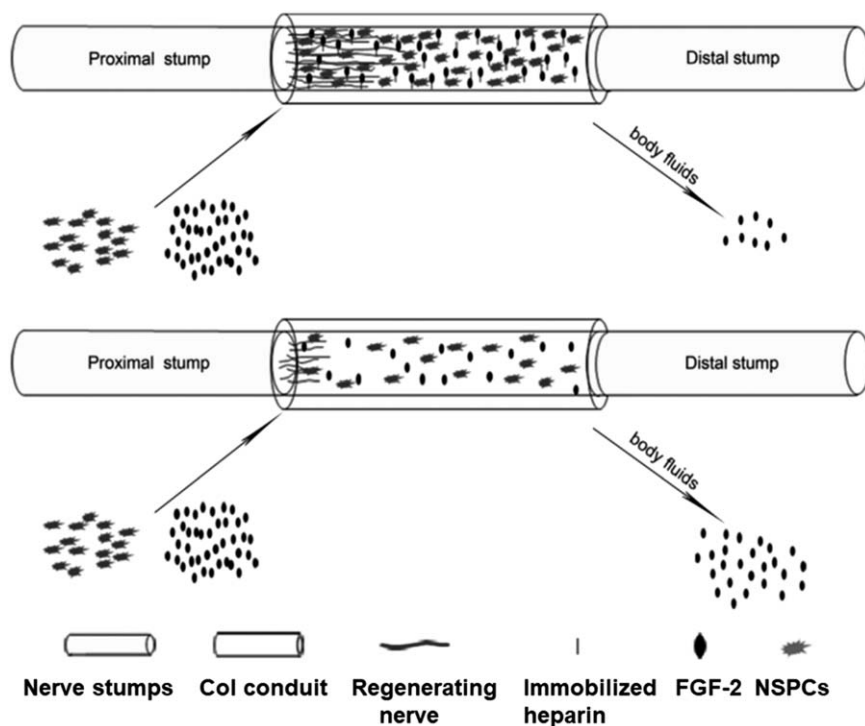


Figure 2.4 Schematic diagram showing HS-bFGF-COL (a) and COL nerve conduits containing NPS cells.¹⁰³
Adapted from ref. 103 with permission from Elsevier, Copyright 2017.

2.3.3 Scaffolds Using Collagen Type II and Type III

COL type I is used for cultivation and scaffold biomaterials, which facilitate osteogenic induction of MS cells by mimicking the bone environment. Although COL type II would be the preferred biomaterial for scaffolds that facilitate chondrogenic induction, only COL type I was accepted for clinical use by the FDA, and COL type I is much less costly than COL type II. Thus several researchers have examined the chondrogenic induction of MS cells in COL type I hydrogels.

It is extremely hard for MS cells to induce into chondrocytes in 2D monolayer cultivation. Hanging drop culture and pellet culture of MS cells are the standard methods for chondrogenic induction of MS cells.¹¹³ High inoculating concentration facilitates higher chondrogenic induction, suggesting that cell-cell interaction and autocrine growth factors are key points in the chondrogenic differentiation. The cluster of MS cells starts the initial chondrogenic differentiation during skeletal development,¹²⁹ which provides the reason for chondrogenic differentiation using pellet cultivation with high density.^{49,130} *N*-cadherin inhibition, a cell-cell adhesive molecule temporarily elevated during chondrogenesis, was observed to obstruct cell

clustering and *in vitro* chondrogenic gene expression.^{49,131} Furthermore, cell morphologies in pellet culture and hanging drop are found to be round and not to spread, as they do in monolayer cultivation. Morphological control is another major point, which facilitate chondrogenesis of MS cells.

Bosnakovski and his colleagues examined chondrogenic induction of bovine BMS cells in several hydrogels in comparison to TCP dishes (monolayer cultivation).⁵⁴ BMS cells were cultivated in COL type II, COL type I, and alginate hydrogels. The chondrogenic induction marker genes, cartilage oligomeric protein, aggrecan, COL type II, and Sox9, were enhanced in COL type II and COL type I hydrogels. No significantly different expression of these chondrogenic induction genes was observed between each COL hydrogel, whereas these genes showed extremely low expression by cells in 2D cultivation.⁵⁴ Chondrogenic induction of BMS cells in both COL type II and type I was found to be better than that in alginate hydrogels from chondrogenic gene expression, although chondrogenic induction in alginate hydrogels was better than that on 2D cultivation. This result shows that both COL type II and type I are appropriate biomaterials for chondrogenic induction of BMS cells. Importantly, the chondrogenic gene expression in BMS cells in COL type II and type I hydrogels in proliferation media was not greatly different from the gene expression of cells in chondrogenic media (supplemented with dexamethasone and TGF- β 1) in this research.⁵⁴ Cells adopted a plump and round morphology and did not spread out in hydrogels. Therefore, both the biological interaction between COL and cells and the physical space effects, which guide the round shape of BMS cells, facilitate chondrogenic induction of BMS cells.⁵⁰ The expression of the COL type I gene is an index of de-differentiation of chondrocytes.⁵⁴ The expression of the COL type I gene that was more or less high in expansion media, could be restricted in BMS cells in COL hydrogels using chondrogenic differentiation media with dexamethasone and TGF- β 1.⁵⁴

In brief, BMS cells cultivated solely in COL type I hydrogels or scaffolds cannot be induced into osteoblasts in a medium with no supplement (BMP-2, ascorbic acid, and/or dexamethasone). On the other hand, hydrogels consisting of COL type II and type I can show chondrogenesis in a medium with no supplement. Chondrogenesis of BMS cells in COL type II hydrogels appears to be higher than in COL type I. COL type II is the predominant molecule of hyaline cartilages. Chondrocyte connects to COL type II *via* $\alpha_{10}\beta_1$, $\alpha_2\beta_1$, and $\alpha_1\beta_1$ integrins that enhance the generation of signaling complexes for response to mechanical stimulation, cell survival, matrix remodeling, and differentiation.^{54,132} Mitogen-activated protein kinase has a significant function in mediation of the downstream signals from integrins, and it can control gene expression *via* stimulation of transcription factors such as AP-1 and NF κ B.

Lu and his colleagues examined whether COL type II suits chondrogenic differentiation by effecting cell morphology *via* Rho A/Rock signaling and β 1 integrins using ADS cells trapped into COL type II and type I hydrogels.⁵⁰ Several things were observed: (1) β 1 integrin inhibition restricted the

differences in gene expression of chondrocytes and also reduced the differences in gene expression of Rock 2 and Rock 1 and cell morphology in comparison to ADS cells in COL type II and type I hydrogels.⁵⁰ (2) ADS cells in COL type II hydrogels indicated lower Rock 2 expression and a rounder shape than the cells in COL type I hydrogels in proliferation media. (3) ADS cells in COL type II hydrogels indicated more effectively chondrogenic differentiation and better expression of chondrocyte genes (aggrecan, Sox9, Sox6, COL type X, and COL type II) than those in COL type I hydrogels, when cells were cultured in expansion medium and chondrogenic induction medium.⁵⁰ We surmise that COL type II produces the initial signals for chondrogenic induction in ADS cells by facilitating a round cell morphology *via* $\beta 1$ integrin-mediated Rho A/Rock signaling.⁵⁰

COL type II is a promising chondrocyte-inductive matrix for BMS cells and is an important component of cartilage ECM, although the application of COL type II is limited by deficient gelation and fibrillogenesis. Therefore, Yang and his colleagues synthesized COL type II grafted with methacrylamide (COL-II-MA) using an amidation reaction between the ϵ -amino groups on COL lysine and methacrylic anhydride, which enables photo-crosslinking of the COL (Figure 2.5).¹⁰⁶ Rabbit BMS cells entrapped in the COL-II-MA hydrogels exhibited facilitation of proliferation and morphological changes that are similar to chondrocytes and the expression of chondrogenic genes were upregulated. Furthermore, extensive secretion of the cartilaginous matrix was found in BMS cells entrapped in the COL-II-MA hydrogels.¹⁰⁶ The results suggested that this effective synthetic approach accelerated the formation of photoactive COL type II hydrogels with a preserved triple helical conformation, which leads to BMS cells with an appropriate niche for proliferation and the essential chondrocyte-inductive matrices for differentiation.

A combination of COL type III and type I, which are plentiful proteins in the osteocyte site, is osteoinductive, and hybrid sponges composed of COL type III and type I have been used for MS cell cultivation biomaterials.^{40,133–135} Schneider and his colleagues examined the osteogenic induction of UC-MS (perinatal MS cells from Wharton's jelly of the umbilical cord) cells and BMS cells in a hybrid scaffold of COL type III (10%) and type I (90%).⁴⁰ As a result of their primitive state, UC-MS cells were considered to have a greater induction ability than BMS cells, which do not express ES cell markers (Nanog and Oct4). However, UC-MS cells showed a poor potential to induce into adipocytes in 3D cultivation as well as in monolayer cultivation.^{40,136,137} Moreover, BMS cells showed the highest extracellular mineralization and osteogenic induction when the cells were cultivated under osteogenic conditions in 2D culture. On the other hand, UC-MS cells in a hybrid scaffold of COL type III and type I exceeded BM-MS cells in ECM protein production.⁴⁰ BMS cells and UC-MS cells showed all the characteristics required for appropriate bone fracture cure *in vivo*. The expression of ECMs is very different in the two cell types, indicating different strategies for bone generation.

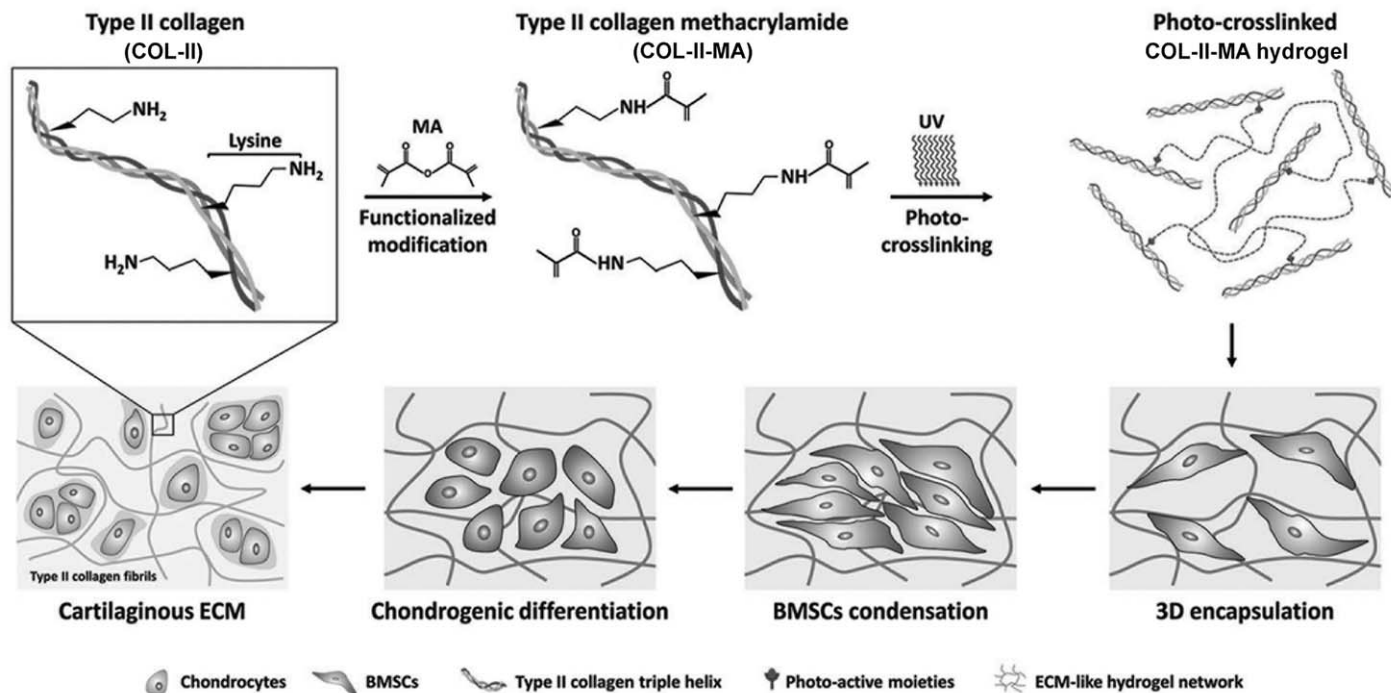


Figure 2.5 Design of one-step crosslinked COL type II (COL-II-MA) for chondrogenic differentiation of rabbit BMS cells. COL type II is selectively functionalized by modification with photoactive moieties of methacrylamide (MA). Photo-crosslinking in the presence of UV irradiation successfully leads to the formation of COL type II hydrogels, which can encapsulate BMS cells within a 3D microenvironment and induce further chondrogenic differentiation.¹⁰⁶ Adapted from ref. 106 with permission from The Royal Society of Chemistry.

Nucleus pulposus (NP) degeneration is commonly the origin of intervertebral disc degeneration and consequent lower back pain. ADSC-based therapy is considered to be a promising treatment of degenerated NP.¹⁰⁷ However, there is a lack of viable cell carriers for transplantation of ADS cells into the NP of patients while supporting cell function. Therefore, Zhou and his colleagues developed a COL type II/chondroitin sulfate (CS) composite hydrogel for the ADS cell (COL-CS-ADSC) delivery system, which was cross-linked with genipin, because the main component of the NP is COL type II and CS.¹⁰⁷ The induction effect of the scaffold on ADS cell differentiation was investigated *in vitro*, and a rat degeneration model of coccygeal vertebrae was used to study the regenerative effect of the COL-CS-ADSC system on the degenerated NP *in vivo*. The results suggested that the COL-CS-ADSC delivery system crosslinked with 0.02% genipin was biocompatible and promoted the expressions of NP-specific genes.¹⁰⁷ ECM synthesis, water content, the disc height, and structure of the degenerated NP were found to be partly restored after injection of the COL-CS-ADSC system into the rat model.¹⁰⁷ The COL-CS-ADSC delivery system used minimally invasive approaches to improve the regeneration of degenerated NP and provided a new avenue for the treatment of degenerative disc disease.

2.3.4 Hybrid Collagen Scaffold Using Inorganic Materials

The main components of human bone are organic COL type I and inorganic hydroxyapatite (HYA, a natural ceramic). Furthermore, there are small quantities of ground substances, such as velum lipids, proteoglycans, and glycoproteins that have been proven to play major roles in controlling mineralization and bone regeneration.^{79,138}

Native bone is made of nano-sized carbonate-substituted HYA (nano-HYA) crystals in COL networks. The preparation of scaffolds closely mimicking the microstructure and composition of COL and nano-HYA in bone should be valuable for osteogenic induction of BMS cells.⁴⁹ Some investigators have indicated that HYA facilitates induction of MS cells into osteoblasts.^{66,94,139,140} Dawson and his colleagues made COL-HYA scaffolds using the following processes: HYA solution was mixed with a COL solution. Then, the solution was frozen at -30°C or -80°C . The frozen COL-HYA solids were then dried. Critical point drying using liquid carbon dioxide led to a dry sponge scaffold (Figure 2.6).⁴⁹ Primary hBMS cells were inoculated onto the COL-HYA scaffold and following 3 days of differentiation into osteoblasts, these were subcutaneously transplanted into immunodeficient mice. After 28 days, the transplanted cell-scaffolds became a slightly compact shape in the subcutaneous cavities and were encircled by host neo-vasculature.⁴⁹ The COL-HYA scaffold was extensively merged with the host tissues, and high cell migration into the scaffold was detected. New osteoid matrices were proved by the characteristic properties of cells embedded in lacunae within the birefringence of organized COL fibers and the matrices under polarized light.⁴⁹ Furthermore, COL-HYA scaffolds inoculated with hBMS cells and

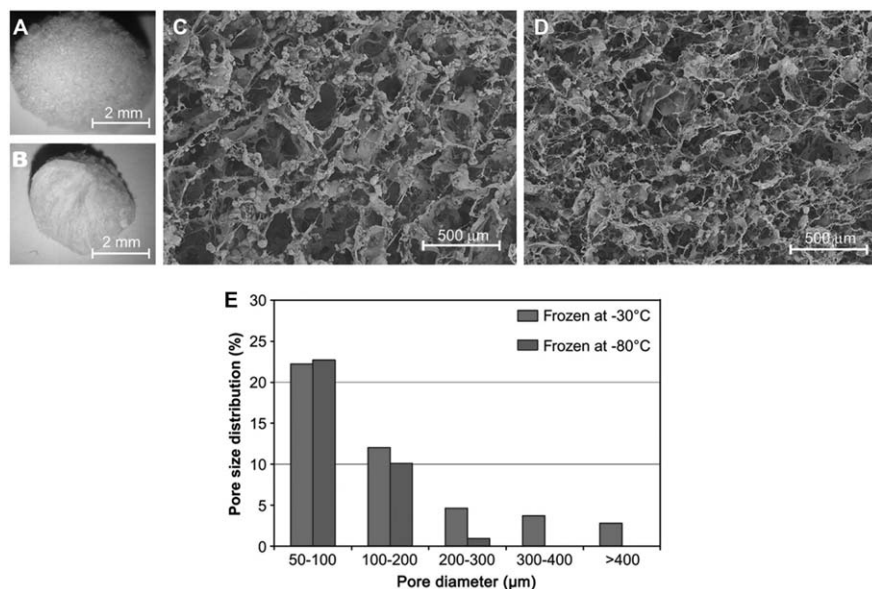


Figure 2.6 Morphology and microstructure of COL and COL-HYA scaffolds. Scaffolds of approximately 4–6 mm in diameter composed of COL and hydroxyapatite (HYA) (A) or COL only (B) were prepared by critical point drying allowing controllable porosity. SEM of a cross-section of COL-HYA scaffolds prepared by freezing at -30°C (C) or -80°C (D). Pore size distribution of COL-HYA scaffolds generated at different freezing temperatures (E).⁴⁹ Adapted from ref. 49 with permission from Elsevier, Copyright 2008.

cultivated for 2 days in osteogenic induction were transplanted subcutaneously in immunodeficient mice on devitalized mice femur with segmental 'v'-shaped defects. Transplanted cell-scaffolds showed excellent integration with mouse femurs, as proven by high areas of deposited matrices surrounding the entrapment of the femur edges and damaged site. Therefore, COL-HYA scaffolds can promote osteogenesis *in vivo*. Both HYA and COL promote the osteogenic response in COL-HYA scaffolds entrapped with MS cells. It is thought that COL-nanocrystalline HYA scaffolds or COL-HYA have higher osteoconductive characteristics than COL or HYA alone.^{49,66,94,140–142}

Bioactive glasses (BGs) such as $\text{CaO-P}_2\text{O}_5\text{-SiO}_2$ are similar to native inorganic compositions of bone and have been shown to activate the generation of calcium phosphates from physiological solution, which results in increased strength at the bone-matrix interface.^{79,143} Composite biomaterials comprised of COL type I and a BG have been examined as bone regeneration matrices.^{79,144} Matrix vesicles, extracellular lipid bilayer-enclosed microstructures produced by calcifying cells, have been shown to start mineral generation during bone formation.^{145,249} In particular, PPS (phosphatidylserine) has good interaction for Ca ions and might be a valuable material of newly generating bone.^{146,147} Xu and his colleagues

investigated a biomimetic composite scaffold of BG-COL-PPS (bioglass-COL-phosphatidylserine) using a freeze-drying method.⁷⁹ The BG-COL-PPS composite scaffold was composed of 35 wt% organic materials and 65 wt% inorganic materials, where the organic material was comprised of 20% PPS. BMS cells and 80% COL type I in the BG-COL-PPS composite scaffold showed a greater degree of osteogenic differentiation, growth and cell adhesion than those on the BG-COL scaffold *in vitro*, which was evaluated by Alizarin Red staining, osteogenic gene expression (osteocalcin, osteopontin, and ALP), ALP activity, and dsDNA content.⁷⁹

BG-COL-PPS scaffolds inoculated without and with rat BMS cells were transplanted in rat femur defects to study *in vivo* osteogenesis and biocompatibility of the scaffold.⁷⁹ BG-COL-PPS scaffolds showed extensive osteoconductivity and high biocompatibility with host bone. BG-COL-PPS with BMS cells greatly increased the efficiency of new bone generation in comparison to BG-COL with BMS cells or BG-COL-PPS without BMS cells.⁷⁹ This research indicates the importance of PPS in COL-bioglass hybrid scaffolds for inducing increased bone generation.

2.3.5 Collagen Scaffolds Immobilized Antibody Targeting Stem Cells

Mobilized stem cells are not able to be significantly recruited into the damaged domains in the body, although some stem cells are identified to be circulating in the bodies.⁴² In heart failure, engineered cardiac patches consisting of ECMs have been used to cure heart damage, although myocardial treatment was restricted because of the low ability for stem cell infiltration.^{42,148,149} A recent strategy where stem cells are recruited from the circulation system using matrices or patches with immobilization of ligands or antibodies, which catch specific stem cells, was examined by Shi and his colleagues (Figure 2.7).⁴² They prepared collagen membranes and scaffolds grafted with anti-Sca-1 monoclonal antibodies using sulfo-SMCC (sulfo-succinimidyl-4-[*N*-maleimidomethyl] cyclohexane-1-carboxylate) and Traut's reagent.⁴² Sca-1 is a typical marker for adult murine hematopoietic stem cells, which is a member of the Ly 6 family. Furthermore, Sca-1 positive (Sca-1⁺) cells obtained from heart and skeletal muscle were known to be multipotent stem cells.¹⁵⁰ Shi and his colleagues tried to enhance autologous stem cells at wound domains using a stem cell-catching COL scaffold combined with Sca-1 antibodies in mice.⁴² The antibody-immobilized scaffolds were transplanted in the hind leg muscle (Figure 2.7E).⁴² Sca-1⁺ cells were observed to be enriched three-fold in the scaffold combined with anti-Sca-1 monoclonal antibodies than in the scaffold with no antibodies. When the functional COL scaffolds were implanted into mice as a cardiac patch to cure surgical heart defects, more cells and capillaries infiltrated transplants with combined anti-Sca-1 antibodies.⁴² Three months after surgical operation, the revival of cardiomyocytes was detected in antibody-combined

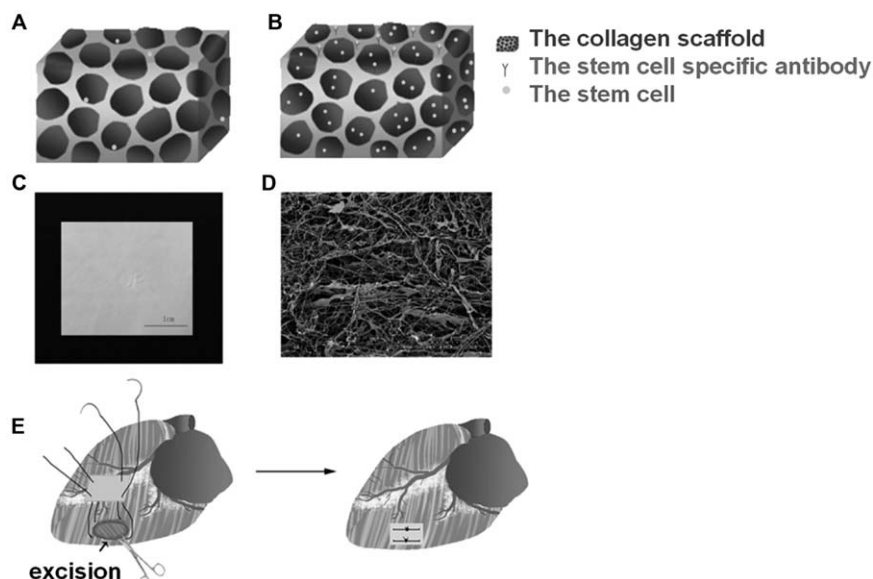


Figure 2.7 The COL scaffold and the schematic diagram for stem cell capture. (A) Few stem cells are retained on the COL scaffold without any modification. (B) The COL scaffolds having specific stem cell antibodies could capture more stem cells. (C) Macroscopic picture of COL scaffolds. Scale bar = 1 cm. (D) The SEM picture of COL scaffolds. Scale bar = 100 μm . (E) The surgery procedure of cardiac patch.⁴² Adapted from ref. 42 with permission from Elsevier, Copyright 2008.

cardiac patches, while COL remodeling and tissue regeneration was delayed in control cardiac patches. COL scaffolds immobilized with ligands or antibodies targeting special stem cells should be another powerful strategy for assembling and recruiting stem cells at injury domains.

2.3.6 Differentiation into Endoderm and Ectoderm Lineages Using Collagen Scaffolds

Hydrogels and scaffolds consisting of COL are primarily used in regenerative medicine for chondrogenic and osteogenic induction of MS cells. However, COL scaffolds have also been used for endodermal and ectodermal induction of MS cells.^{39,91,92}

PLCL, poly(L-lactic acid-co-3-caprolactone), which is a biodegradable and synthetic polymer and a non-toxic copolymer of PCL, poly(ϵ -caprolactone), and PLLA, poly-L-lactic acid, has been studied as a material for drug delivery systems and surgery.^{43,151} On the other hand, COL is a native ECM protein with great cell adhesive characteristics but not great mechanical strength. Prabhakaran and his colleagues made electrospinning nanofibers by mixing PLCL and COL, which enhanced its biocompatibility while maintaining mechanical strength and produced a hydrophilic mesh with small fiber

diameter and high porosity, which are preferable for nerve tissue engineering.⁴³ MS cells differentiated on PLCL/COL type I nanofiber scaffolds expressed nestin and NF200 (neurofilament) protein, as examined by immunofluorescent labeling, and also displayed neuronal morphologies with multipolar elongations.⁴³

The mammalian CNS has little ability for self-repair after damage, and neurons do not expand. As a result, neural tissue engineering using hydrogels inoculated with neural stem cells might generate options for cure of injured CNS tissues. Ma and his colleagues made COL type I hydrogels inoculated with neural stem cells extracted from embryonic rat subcortical or cortical neuroepithelium and cultivated neural stem cells in serum-free media.³⁹ The COL-entrapped stem cells proliferated and efficiently created neurons, which exhibited excitability, ion channels/receptors, neurotransmitters, and neuronal polarity.³⁹ The differentiation from BrdU⁺/Tuj1⁻ to BrdU⁻/Tuj1⁺ cells was followed by shifts in the expression of valuable receptors for neurotransmitters from purinergic and cholinergic states to glutamatergic and GABAergic states.³⁹ Spontaneous post-synaptic currents were examined by a patch-clamp method from stem cell-differentiated neurons. These findings indicate that neural stem cells cultivated in COL hydrogels recapitulate development of CNS stem cells.

2.4 Gelatin

Gelatin is heat-denatured COL that is a combination of proteins and oligopeptides made by partial hydrolysis of COL derived from the intestines, organs, connective tissues, and boiled bones of animals.¹⁵² Gelatin is a varied mixture of multi-stranded or single oligopolypeptides comprising between 300 and 4000 amino acids. Two typical kinds of gelatin, Type B and Type A, are known.¹⁵² Type A gelatin is derived and treated by acidic treatment of COL, while Type B gelatin is processed by alkaline treatment.¹⁵² The alkaline treatment changes asparagine and glutamine residues into aspartic and glutamic acids, respectively, which generate a larger carboxylic acid amount for Type B gelatin than for Type A gelatin. Gelatin has some good points over other native proteins, such as its low cost, commercial availability, biodegradability, and biological origin.¹⁵² Gelatin dissolves to an aqueous solution at elevated temperatures and solidifies when cooled. As a result, it is simple to make hydrogels and to hold stem cells in gelatin. The chemical component of gelatin is close to that of its parent COL. Table 2.5 shows some of the types of gelatin biomaterials or scaffolds for MS cell differentiation described by several researchers.^{67,152–161}

2.4.1 Gelatin Hydrogels and Scaffolds

Ponticello and his colleagues used a porous gelatin sponge, Gelfoam[®] (used as a hemostatic agent), as a delivery method for hMS cells in cartilage development therapy. hMS cells in Gelfoam[®] generated cartilage-like ECMs

Table 2.5 Investigations into stem cell induction on gelatin biomaterials in 2D and 3D culture.¹ Reproduced from ref. 1 with permission from the American Chemical Society, Copyright 2012.^a

Stem cell source	Material for stem cell culture	Differentiation	Ref.
hBMSCs	Gelatin (2D culture, coating on dishes)	Osteoblasts	92
hBMSCs	Gelatin/HA (2D culture, hydrogel particles)	Osteoblasts	153
hBMSCs	Gelatin (2D culture, coating on dishes)	Pancreatic cells, neural cells, osteoblasts, adipocytes	154
Rat BMSCs	Gelatin (3D culture, scaffold)	Osteoblasts	155
Rat BMSCs	Gelatin (3D culture, microparticles)	Osteoblasts	156
hADSCs	Gelatin (3D culture, scaffold)	Chondrocytes	159
rBMSCs	Gelatin/esterified HA (3D culture, scaffold)	Chondrocytes	157
hBMSCs	Gelatin (3D culture, scaffold)	Cartilage	158
mESCs	Gelatin/PCL (3D culture, nanofibers)	Cardiac progenitors	161
hADSCs	Gelatin/PCL (3D culture, electrospinning mat), gelatin/collagen I/PCL (3D culture, electrospinning mat)		152

^aADSCs, adipose-derived stem cells; BMSCs, bone marrow stromal cells; HA, hyaluronic acid; hADSCs, human ADSCs; hBMSCs, human BMSCs; PCL, poly(ϵ -caprolactone); rBMSCs, rabbit BMSCs.

including COL type II and sulfated glycosaminoglycans after 3 weeks of culture *in vitro*.¹⁵⁸ Gelfoam[®] cylinders entrapping hMS cells were found to be biocompatible, without evidence of lymphocytic infiltration or immune response at the site of transplantation in osteochondral defects in the rabbit femoral condyle. Gelfoam[®] resorbable gelatin matrices would be a valuable candidate as vehicle matrices for tissue engineering using hMS cell-based cartilage.¹⁵⁸

Chondrogenic induction of human ADS cells in agarose and alginate hydrogels and in gelatin scaffolds (Surgifoam[®]) was investigated by Awad and his colleagues.¹⁵⁹ hADS cells in gelatin scaffolds displayed highly polygonal shapes, while cells entrapped in agarose and alginate hydrogels showed spherical morphologies. Extensive cell-mediated contraction of the gelatin scaffolds was detected, with a decrease of up to 87% and 70% of their original diameters under control and chondrogenic cultivation conditions, respectively, whereas agarose and alginate scaffolds entrapping cells show no contraction.¹⁵⁹ Proteoglycan and protein synthesis spread in the gelatin scaffold was much higher than in alginate (68%) and agarose (30–32%) on day 1.¹⁵⁹ The cell number in gelatin scaffolds was 40–50% higher than in

alginate and agarose scaffolds on days 14 and 28. The content of hydroxyproline and sulfated glycosaminoglycan increased greatly (by 2.5- to 9-fold) between days 1 and 28 for any sponges entrapping cells, which were cultured in chondrogenic induction medium.¹⁵⁹ Gel contraction was developed in the domains enhanced in COL type II and CS that imply cartilage formation. The agarose hydrogels and gelatin scaffolds held shear modulus three times higher than alginate hydrogels. It should be noted that the shear and compressive modulus of the hydrogels and scaffolds were of the order of 5% or less than those of natural cartilage.^{159,162,163} The enhancement in shear modulus was seen to be strongly related to enhancement in contents of hydroxyproline and sulfated glycosaminoglycan. Gelatin is an interesting material for scaffolds of hADS and hMS cells. However, it is important to design in the future gelatin-based scaffolds entrapping hADS or hMS cells that have approximately the same shear and compressive moduli as natural cartilages.¹⁵⁹

Payne and his colleagues developed a degradable, *in situ* crosslinkable and injectable gelatin carrier for MS cells. MS cells were entrapped in uncrosslinked gelatin microparticles having an average diameter of 630 μm , each including around 50 cells.¹⁵⁶ Gelatin microparticles were crosslinked with shell thickness of 75 μm from exposure to dithiobis(succinimidylpropionate) (DSP) solution. MS cells could proliferate in crosslinked and uncrosslinked gelatin microparticles, which retained their proliferative ability and osteoblastic phenotype over 4 months.¹⁵⁶ The entrapment of MS cells in microparticles crosslinked with DSP is effective for temporary protection of the cells from toxic local locations.¹⁵⁶

MS cells are prepared by seeding cells from BM (bone marrow) or other sources in TCP flasks and by selection of plastic-adhesive cells with fibroblastoid morphologies. Battula and his colleagues purified MS cells from non-amniotic placenta (PL) and BM by cultivating Ficoll-selected cells in gelatin-coated plates in serum-free media supplemented with FGF-2 that was used for hES cell proliferation.¹⁵⁴ MS cells selected in gelatin-coated plates in hES cell media displayed a 4- to 5-fold higher expansion rate than typically prepared MS cells, which were cultivated on TCP plates in serum-containing media. On the other hand, the colony-forming unit fibroblast (CFU-F) number was found to have only a 1.5- to 2-fold enhancement in PL-MS cells and has no effect in BM-MS cells. BM-MS and PL-MS cells cultivated on gelatin-coated plates in hES cell media exhibited enhanced marker expression of the pluripotent stem and progenitor cells, nestin, nanog-3, frizzled-9 (FZD-9), Oct-4, and SSEA-4. PL-MS cells showed higher expression of FZD-9, SSEA-4, and Oct-4 than BM-MS cells.¹⁵⁴ However, BM-MS and PL-MS cells cultivated on TCP plates expressed a greatly reduced intensity of nestin, Oct-4, and SSEA-4 than the cells cultivated on gelatin-coated plates. There was no expression of nanog-3 and FZD-9 in PL-MS cells and BM-MS cells cultivated on gelatin-coated plates. The MS cells cultivated on gelatin-coated plates had multi-lineage induction abilities, as shown by their ability to generate cells of endodermal (pancreatic-like) and ectodermal

(neuron-like) differentiation lineages, as well as mesodermal lineages (adipocytes and osteoblasts).¹⁵⁴ Remarkably, the CFU-F capacity of PL-MS and BM-MS cells was not greatly changed by the different cultivation conditions, indicating that the stem cell pool of MS cells was not changed. Battula and his colleagues suggested that FZD-9 might be an index of primitive MS cells, which would identify them from adult MS cells, and can be elucidated by the fact that Wnt-FZD9 signaling is necessary for stem cell expansion.¹⁵⁴

The ideal ECM for choosing primitive MS cells by cultivation of adipose tissue, amniotic fluid, and BM on ECM-grafted or ECM-coating biomaterials has not yet been established and would be a vital investigation project for material investigators in future. Adequate ECM-grafted or ECM-coated surface could sort cells with greater quantities and higher pluripotency of primitive MS cells in comparison to gelatin-coating or TCP plates.

Crosslinked gelatin gels by photo-initiation, which entrapped with chondrocytes has also been evaluated.¹⁶⁴ The gelatin molecules were grafted with methacrylic acid to generate crosslinkable gelatin, which generated chemically crosslinked hydrogels by photo-initiated polymerization. The gelation time was simply adjusted and had an inverse correlation with gelatin concentration. The double carbon bonds were not detected in the gels from evaluation of the proton spectra of nuclear magnetic resonance assay.¹⁶⁴ The loss modulus and storage modulus of the gels were found to increase with higher gelatin concentrations, whereas the mesh size and swelling ratio were found to decline.¹⁶⁴ TGF- β 1 was also included in the gelatin hydrogels to enhance their bioactivity.¹⁶⁴ The chondrocyte cultivation *in vitro* indicated that the gelatin hydrogels had superior potential in supporting the chondrocytic phenotype and maintaining chondrocyte growth. Immobilization of TGF- β 1 was found to further enhance the biological activities in terms of both cell expansion and ECM secretion.¹⁶⁴

2.4.2 Gelatin Hybrid Scaffolds

Gelatin is known to be a brilliant biomaterial for stem cell differentiation, proliferation, and attachment.^{157,165–167} However, the shortcomings of using gelatin as scaffolds in regenerative medicine are its rapid biodegradation and insufficient biomechanical stiffness.^{157,166} Esterified HA has a longer lifetime in the biopolymer matrices. The scaffolds made from esterified HA allows sufficient time for them to be used as *in vivo* biomaterials for induction of MS cells and matrix generation. On the other hand, esterified HA-based surfaces can hinder cell adhesion,^{157,165} and MS cells on the surface are noted to (re)differentiate *in vitro*.¹⁵⁷ Hyaff11, a pure HA benzyl ester, was known to promote degradation by hydrolysis of the ester bond in 3–5 months *in vivo*^{157,168} and in 60 days *in vitro*.^{157,169} Cell-loaded gelatin scaffolds were known to dissolve after 7–14 days *in vivo*^{157,170} and after 10 days in *in vitro* cultivation because of collagenolytic potential of infiltrating cells.^{157,168}

Angele and his colleagues examined the potential of composite scaffolds composed of gelatin (30%) and esterified HA (Jaloskin[®], 70%) to promote the induction of rabbit BMS cells to engineer bone and cartilage. The composite scaffold was formed by a salt leaching method.¹⁵⁷ The composite scaffold had pores with two distinct size ranges, 250–500 μm and 50–150 μm in diameter, and possessed a few blind-ended pores and majorly interlinked pores. Cell-loaded and empty composite scaffolds were cultured for up to 4 weeks in the medium without and with TGF- β 1. A COL type II-rich ECM was generated by cells inoculated in the composite scaffolds and cultivated in the medium supplemented with TGF- β 1.¹⁵⁷ The composite scaffold promoted osteochondrogenic induction of rabbit BMS cells when the scaffolds were transplanted subcutaneously into immunodeficient mice, while no osteochondral induction was detected in transplanted composite scaffold with no cell.¹⁵⁷ *In vitro* pre-culture in chondrogenic media enhanced the percentage of osteochondral tissue in the composite scaffold after 21 days *in vivo*. These outcomes show that these composite biomaterials could be valuable in regenerative medicine.¹⁵⁷

Takahashi and his colleagues developed biodegradable gelatin sponges including different amounts of β -tricalcium phosphate (gelatin- β TCP)¹⁶⁰ and examined the osteogenic induction of MS cells taken from rat BM *in vitro*. The gelatin sponges including β TCP had an interconnected pore morphology with an average size around 180–200 μm , which did not depend on the amount of β TCP.¹⁶⁰ The rigidity of the sponges was higher with increasing amounts of β TCP. MS cells were homogeneously distributed throughout the sponge when the cells were inoculated by agitation. The morphologies of cells adhered on the gelatin- β TCP showed more spread with the larger amount of β TCP.¹⁶⁰ The rate of MS cell expansion was dependent on the amount of β TCP and the cultivation protocol: the higher the proliferation rate, the more β TCP in the stirring cultivation. The degree of deformation of the gelatin- β TCP sponges was minimized with increasing amount of β TCP. The osteocalcin content and ALP activity, as markers of osteogenic induction, were highest for the sponges with a β TCP amount of 50 wt%.¹⁶⁰ The osteocalcin content and ALP activity were observed to be meaningfully higher in stirring cultivation in comparison to those in static cultivation. Therefore, the osteogenic differentiation, proliferation, and attachment of MS cells are affected by the composition of β TCP and gelatin sponges.

Electrospinning using native ECMs is a feasible skill for the manufacture of fiber scaffolds for several uses in regenerative medicine. One disadvantage of scaffolds electrospun from native ECMs is the need to use a crosslinking agent for stability, which is considered to lead to several problems *in vivo*, such as graft failure. Glutaraldehyde has typically been used as a crosslinking agent for electrospinning COL-based nanofibers.^{171–174} Glutaraldehyde was necessary for intermolecular crosslinking of the fibers in the scaffold for cell cultivation to avoid dissolution in cultivation media. The crosslinked scaffold displayed noticeably thickened fibers, which frequently

fused into one another, and the porosity reduced significantly, making them inappropriate scaffolds for 3D cultivation of stem cells. In addition, residual glutaraldehyde is actually toxic to stem cells and tissue.¹⁵²

Heydarkhan-Hagvall and his colleagues fabricated hybrid nanofibrous scaffolds of PCL and gelatin and hybrid nanofibrous scaffolds of PCL, elastin and COL using an electrospun method with no toxic crosslinking agent.¹⁵² Electrospinning PCL/gelatin scaffolds produced a higher tensile strength compared to PCL/elastin/COL nanofiber scaffolds. PCL doping in the ECM solution allowed the electrospun solution to generate self-standing scaffolds in aqueous conditions. It is important to increase the PCL concentration to a minimum of 5% in the scaffold to preserve their 3D porous morphologies without the use of a crosslinking agent.¹⁵² Both hybrid scaffolds were inoculated with ADS cells to examine the effects of pore size on cell migration and adhesion. Perfect cell adhesion was found on the surfaces of both hybrid scaffolds. It was discovered that cell infiltration into the scaffold was extensive in the PCL/gelatin hybrid scaffolds.¹⁵² The combination of 10% gelatin with 10% PCL demonstrated much higher tensile strength in comparison to COL or gelatin and elastin alone, and this resulted in a pliant and uniform fibrous mat.¹⁵² It is concluded that electrospinning of hybrid scaffolds with synthetic polymers and natural proteins can be used to manufacture tissue-engineered scaffolds, which can replicate important properties of the native ECM, including its biochemical and mechanical characteristics. The combination of synthetic polymers and natural proteins to form electrospun fiber morphologies leads to scaffolds with desirable biological and mechanical characteristics.¹⁵²

2.5 Laminin

LN is one of the main glycoproteins observed in the basal lamina that is important for mediating a variety of cellular activities, including differentiation, migration, proliferation, and adhesion. LN is a trimeric protein consisting of an α -chain, a β -chain, and a γ -chain, which hold five, four, and three genetic variations, respectively.¹⁷⁵ LN molecules are assigned in line with their chain composition, *e.g.*, LN-332 includes α 3, β 3, and γ 2 chains (LN-5) and LN-111 contains α 1, β 1, and γ 1 chains (LN-1).¹⁷⁶ LN is widely used as a coating for cell cultivation biomaterials,^{177,178} and LN facilitates stem cell induction into neural cells,^{51,56,90,179–182} cardiococytes,^{52,183,184} and osteoblasts.¹⁸⁵ LN is observed to make direct contact with adult human NS (hNS) cells through basal lamina-like extensions from blood vessels in the subventricular zone.¹⁸⁶ As a result, LN is often used as a coating biomaterial on plates for the cultivation of neural cells.¹⁸⁷ Table 2.6 lists studies of LN-coating scaffolds and plates for MS cell induction.^{25,41,45,51,52,56,62,67,73,75,76,182,183,185,188–197}

Yu and his colleagues examined an effective protocol to prepare proliferative dopaminergic neurons from rat NS cells in the presence of LN, heparin, and FGF-2 *in vivo* and *in vitro*.¹⁹⁰ In this study, neurospheres of rat NS cells were cultivated on plates coated with 1 $\mu\text{g cm}^{-2}$ LN and 0.01% PDL,

Table 2.6 Investigations into stem cell induction on 2D and 3D LN biomaterials.¹
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Stem cell source	Material for stem cell culture	Differentiation	Ref.
hBMSCs	Laminin (2D culture, coating on dishes)	Osteoblasts	67, 197
hBMSCs	Laminin-1 (2D culture, coating on dishes)	Osteoblasts	25
hBMSCs	Laminin-5 (2D culture, coating on dishes)	Osteoblasts	185, 188
hBMSCs	Laminin-5 (2D culture, coating on dishes)	Osteoblasts, chondrocytes	189
hADSCs	Laminin (2D culture, coating on dishes)	Adipocytes	45
hBMSCs	Laminin (2D culture, coating on dishes)	Smooth muscle cells	52
hADSCs	Laminin (2D culture, coating on dishes)	Cardiomyocytes	183
hESCs (TE03, TE06)	Laminin/PDL (2D culture, coating)	Neural cells	51
hBMSCs	Laminin-1 (2D culture, coating on dishes)	Neural cells	56
hBMSCs	Laminin-10/11 (2D culture, coating on dishes)	Neural cells	56
mESCs	Laminin (2D culture, coating on dishes)	Neural cells	73
Rat neural stem cells	Laminin (2D culture, coating on dishes)	Neurons	196
Rat neural stem cells	Laminin (2D culture, coating on dishes)	Dopaminergic neurons	190
Human neural stem cells	Laminin (2D culture, coating on dishes)	Oligogliocytes	41
mESCs	Laminin-332 (2D culture, coating on dishes)	Lung epithelium	192
Mouse hepatic stem cells	Laminin (2D culture, coating on dishes)	Hepatocytes	75
Mouse NPSCs	Laminin (2D culture, blended hydrogels)	None	194
Mouse BMSCs	Laminin-1 (3D culture, conjugating on PEG hydrogels)	Osteoblasts	195
mESCs	Laminin-332 (3D culture, coating on PDDLA, sheet)	Lung epithelium	192
mESCs	Laminin (3D culture, coating on gelatin/PCL nanofibers)	Cardiac progenitors	161
Rat neural stem cells	Laminin (3D culture, coating on PES fiber mesh)	Neural cells	191
Human NPSCs	Laminin (3D culture, blending with fibrin hydrogels)	Neural cells	193

Table 2.6 (Continued)

Stem cell source	Material for stem cell culture	Differentiation	Ref.
hBMSCs	Laminin (3D culture, coating on PLGA microcarrier)	Dopamine secreting neurons	182
hBMSCs	Laminin (3D culture, coating on PLLA sheet)	Smooth muscle cells	52

^aADSCs, adipose-derived stem cells; BMSCs, bone marrow stromal cells; ESCs, embryonic stem cells; hADSCs, human ADSCs; hBMSCs, human BMSCs; hESCs, human ESCs; mESCs, mouse ESCs; NPSCs, neural progenitor/stem cells; PDDL, poly-DL-lactic acid; PEG, polyethylene glycol; PES, polyethersulfone; PLGA, poly(lactic-co-glycolic acid), PLLA, poly-L-lactic acid.

poly-D-lysine, in cultivation media containing heparin and FGF-2. Most cells remained nestin-positive, which suggests NS cells after 1 day of induction. Neurons were selected from neurospheres, of which a few cells were shown to be GFAP (glial fibrillary acidic protein) positive and some cells showed TH positive (TH⁺, dopaminergic).¹⁹⁰ After induction for a week, more neurons had become dopaminergic cells. Cells primed by heparin and FGF-2 and cultivated on plates immobilized with LN and PDL for a week *in vitro* were implanted into the medial forebrain bundle region and ventral tegmental area of lesioned rats to investigate whether the NS cells were able to be dopaminergic neurons *in vivo*.¹⁹⁰ TH⁺ cells were observed mostly near the injection spots after grafting of 5×10^4 primed NS cells. The combination of heparin and FGF-2 could induce the formation of dopaminergic neurons from rat NS cells cultivated on plates coated with LN and PDL *in vitro* and *in vivo*.¹⁹⁰

Oligodendrocytes are glial cells, which are important for myelin maintenance and formation in CNS, and oligodendrocytes disappear in typical chronic and acute diseases (e.g., multiple sclerosis and Pelizaeus-Merzbacher disease). NS cells obtained from human cord blood cells were noted to undergo oligogliogenesis when NS cells were cultivated on plates immobilized with LN, but not with FN, PLL, or COL type I.⁴¹ The attachment of NS cells to LN facilitated a 2.4-fold enhancement in the oligodendrocyte number (12% on LN *versus* 5% in controls).⁴¹ Matrix metalloproteinase (MMP) expression was also found to increase 3.6-fold on plates coated with LN (2.0% on PLL, 3.0% on FN, 3.6% on LN, and COL type I, 1% in controls), which indicated a link between the activity of metalloproteinases and ECMs, especially LN, in the cells.⁴¹

Tate and his colleagues studied the implantation of FN- or LN-based scaffolds including NS cells into traumatically damaged mouse brain.⁹⁰ Survival of NS cells increased in the LN-based scaffolds in comparison to the FN-based scaffolds. The mice that were transplanted with NS cells in the LN-based scaffold did much better than control (untreated) mice in spatial learning experiments. These results suggest that the selection of appropriate ECMs for the scaffolds loading NS cells would improve cell transplantation therapy.⁹⁰

Ma and his colleagues investigated the effect of ECM proteins on neural induction of hES cells.⁵¹ Embryoid bodies (EBs) formed from hES cells were seeded on plates coated with Matrigel, COL type I, LN/PDL, FN/PDL, and PDL, and cultivated in neural induction media. Neural progenitors and neuronal induction were found to different extents, depending on the biomaterials on which the EBs were cultivated. Neural outgrowth, neuronal formation, and neural progenitor formation were observed to be greatly increased on plates coated with LN-rich Matrigel-coated plates and even LN-coated plates than on other ECM protein-coated plates.⁵¹ LN activated neural outgrowth and expansion of hES cell-derived neural progenitors in a dose-dependent way. The cells from EBs of hES cells attached with LN *via* $\alpha 6 \beta 1$ integrin receptors, which implicates the role of LN/ $\alpha 6 \beta 1$ integrin signaling in guiding neural induction of hES cells.⁵¹

Mruthyunjaya and his colleagues studied the neurite outgrowth induction ability of hBMS cells cultivated on plates coated with LN-10/11, LN-1, COL type IV, COL type I, and FN in the medium containing no growth factors and no differentiation supplement.⁵⁶ Any ECMs examined were observed to maintain attachment of hBMS cells to different degrees. However, only direct interaction with LN-1 started sprouting of neurite-like processes. hBMS cells inoculated on plates immobilized with LN-1 expressed neurites with neurite outgrowth, neuronal morphology, and contracted cell bodies by 24 h.⁵⁶ The interaction of LN-1 with hBMS cells was formed through the MEK/ERK signaling pathway and $\alpha 6 \beta 1$ integrin receptors, because neurite outgrowth was reduced by inhibition of these signals.⁵⁶

Addington and his colleagues developed an implantation carrier to increase the responsiveness of neural transplants to stromal cell-derived factor-1 α (injury-induced SDF-1 α) where SDF-1 α is a potent chemotactic signal, which is readily present after traumatic brain injury.¹⁹⁴ They hypothesized that HA and LN hydrogels promote up-regulation of the expression of the SDF-1 α receptor CXCR4 in NPS cells and promote NPS cell migration in response to SDF-1 α gradients.¹⁹⁴ HA and LN hydrogels (HA-LN, HA-Lm) have been successfully developed. CXCR4 expression on NPS cells and NPS cell chemotactic migration were investigated on HA-LN (HA-Lm) hydrogels. The results indicated that NPS cells significantly increased CXCR4 expression after 48 h of cultivation on the HA-LN (HA-Lm) hydrogels in a manner critically dependent on both LN and HA (Figure 2.8A).¹⁹⁴ Furthermore, NPS cell chemotactic migration significantly increased on the HA-LN (HA-Lm) hydrogels in response to SDF-1 α at 48 h, which was critically dependent on HA, LN and the SDF-1 α gradient (Figure 2.8B).¹⁹⁴ HA-LN (HA-Lm) hydrogels serve to prime NPS cells for the injury microenvironment and provide the appropriate infrastructure to support migration of NPS cells into the surrounding tissue where NPS cells more effectively respond to the injury microenvironment.

Growth factors are significant mediators of osteogenic differentiation of MS cells. However, the use of growth factors in regenerative medicine is not only costly but also may guide unexpected responses in surrounding tissues.

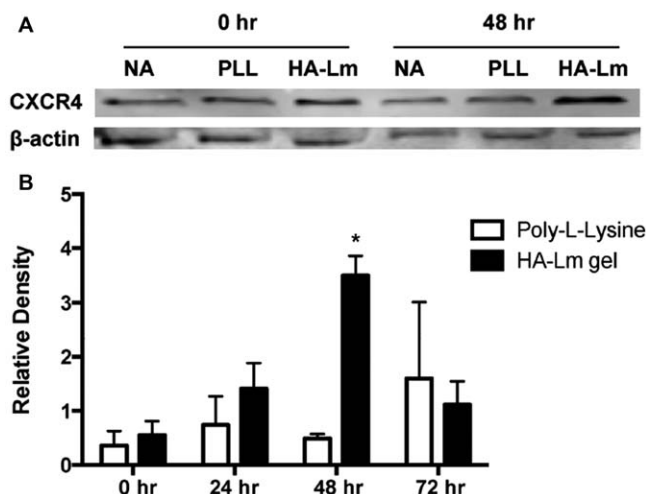


Figure 2.8 HA-Lm (HA-LN) hydrogels promote CXCR4 up-regulation on NPS cells after 48 hours of cultivation. CXCR4 protein expression on NPS cells cultured on HA-Lm hydrogels (Low HA/Moderate Lm (LN)) is significantly increased compared to PLL at any time points and to HA-Lm hydrogels at all other times points in western blotting assay (A, B). CXCR4 expression normalized internally to β -actin expression and externally to CXCR4 expression in non-adherent culture (NA).¹⁹⁴ Adapted from ref. 194 with permission from Elsevier, Copyright 2015.

Therefore, the ability to specifically induce MS cells into osteogenic induction without the use of exogenous growth factors by manipulation of specific scaffold materials would be effective in tissue engineering. Becerra-Bayona and his colleagues investigated the effect of specific ECMs on osteogenic differentiation of murine MS (mMS) cells to develop scaffolds with intrinsically osteoinductive properties.¹⁹⁵ LN-1, FN, and fibrinogen (FG) were selected to investigate ECMs because of their characteristics in bone fracture healing and/or bone morphogenesis. These ECMs were covalently bound into poly(ethylene glycol) (PEGDA) hydrogels and their effects on entrapped mMS cells were investigated. After 1 week of cultivation, mid-term markers of several MS cell lineages were evaluated to examine the strength and specificity of the osteogenic induction potentials. LN-1 conjugated PEGDA (PEG-LN) hydrogels expressed higher levels of osterix (osteogenic transcription factor) compared to the expression at day 0 (Figure 2.9).¹⁹⁵ Furthermore, MS cells cultured on FG conjugated PEGDA (PEG-FG) and PEG-LN hydrogels showed increased deposition of osteocalcin (bone ECM protein) compared to those on PEG-FN gels, as well as the expression at day 0 (Figure 2.9).¹⁹⁵ It should be mentioned that the osteogenic induction of MS cells on PEG-FG and PEG-LN seemed to be specific, because MS cells do not enhance the markers related to chondrocytes, smooth muscle cells (SMCs), and adipocyte lineages compared to the expression at day 0 in these

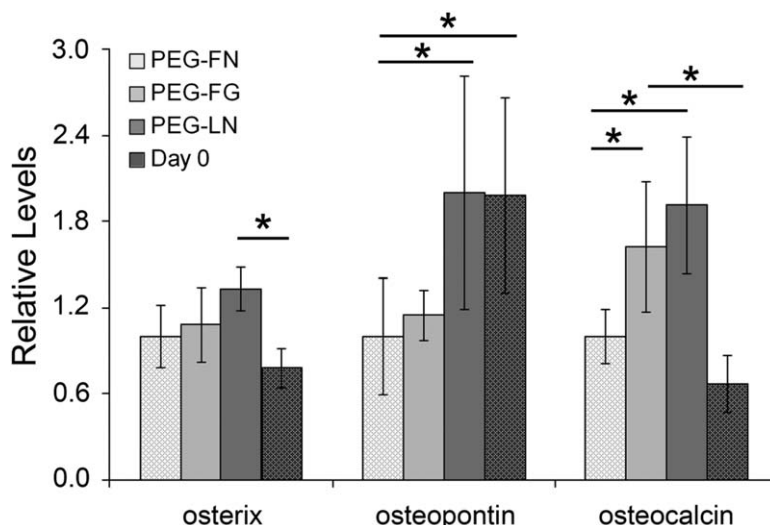


Figure 2.9 Expression of osteogenic markers osteocalcin, osteopontin, and osterix on mMS cells cultured in PEG-FN, PEG-FG, and PEG-LN hydrogels. For the purpose of comparison, expression of each osteogenic marker has been normalized to the corresponding measure for PEG-FN gels.¹⁹⁵ Adapted from ref. 195 with permission from Elsevier, Copyright 2012.

hydrogels. They looked into the integrin dynamics underlying these induction findings, and initial integrin attachment and temporal alterations in cell integrin profiles were examined.¹⁹⁵ The findings indicated that α_6 , α_v , and α_2 integrin subunits play important functions in integrin-mediated osteogenic differentiation.

LN-5 is reported to exist in bone and is also expressed by hBMS cells.¹⁸⁵ hBMS cells produce LN-5 and attach to exogenous LN-5 *via* $\alpha 3\beta 1$ integrin. LN-5 plays an important role in the development of bone tissues by reducing the chondrogenic induction of hBMS cells and by facilitating expansion of hBMS cells.

Klees and his colleagues investigated the fact that the attachment of hBMS cells to LN-5 stimulated ERK within half an hour and guided the phosphorylation of Runx2/CBFA-1 (the osteogenic transcription factor) within 8 days.^{185,188} hBMS cells grown on plates coated with LN-5 for 16 days expressed elevated levels of osteogenic marker genes including osteopontin, osteocalcin, and ALP. Cells grown for 3 weeks produced mineralized matrices, which indicated osteogenic induction.¹⁸⁵ Injection of the ERK inhibitor PD98059 to the cultivation media prevented osteogenic induction of hBMS cells cultivated on plates coated with LN-5 and cells grown on TCP plates in osteogenic induction media. These findings indicated that the interaction of hBMS cells with LN-5, but not with FN, is enough to stimulate ERK and to drive osteogenic induction in hBMS cells in the cultivation media without induction reagents (*e.g.*, dexamethasone).¹⁸⁵

Salaszyk and his colleagues also investigated that the contact of hBMS cells with LN-5 was enough to drive osteogenic induction of hBMS cells *via* an ERK-dependent pathway.¹⁸⁸ It was found that FAK-mediated signaling pathways lead integrin $\alpha 3 \beta 1$ /LN-5 binding and activation of ERK1/2 where LN-5 facilitated osteogenic induction through this signaling pathway.¹⁸⁸

Cardiomyocyte induction of ADS cells cultivated on uncoated, FN-coated, and LN-coated TCP dishes was examined by Dijk and his colleagues.¹⁸³ Expression of an early cardiomyocyte marker, MLC-2a (myosin light chain-2a), was significantly increased in cells on all dishes after 7 days of cardiomyogenic induction, while SERCA2a (late cardiomyocyte marker) was only significantly increased in ADS cells cultivated on LN-coated plates after 5 weeks. The number of desmin-positive cells (a late cardiomyocyte marker) was only significantly increased in ADS cells cultivated on LN-coated dishes. Therefore, human ADS cells cultivated on LN-coated plates can be efficiently induced into cardiomyocytes, specifically during late induction time.¹⁸³

ECMs also play a major function in the phenotypic modulation of SMCs. ECMs might contribute to the induction of MS cells into SM cell lineages. Then, Suzuki and his colleagues examined whether hBMS cells would induce into SM cell lineages for cardiovascular regeneration by cultivation of the cells on plates coated with COL type IV, FN, and LN as well as non-coated plates, in proliferation media containing no differentiation factor (*e.g.*, TGF- $\beta 1$) for a week, and the expression of SM cell-derived proteins and genes was analyzed.⁵² The expression of SM cell-specific proteins and genes (h1-calponin [CALP] and α -smooth muscle actin [ASMA]) in hBMS cells was greatly elevated in cells plated on LN, but not on COL type IV and FN, whereas the number of hBMS cells increased on plates coated with LN, FN, and COL type IV in comparison to non-coated plates.⁵² The LN-coated biodegradable PLLA surface inoculated with hBMS cells were also subcutaneously transplanted in rats. The cells exhibited extremely high expression of CALP and ASMA proteins *in vivo*. The differentiation marker of SM cells (SM2, smooth muscle myosin heavy chain) was detected in hBMS cells on the LN-coated biomaterials by 2 weeks after transplantation.⁵²

Lung epithelial induction of mES cells cultivated on TCP plates and poly-DL-lactic acid plates coated with Matrigel, FN, LN 332 (LN 5), and COL type I was studied by Lin and his colleagues.¹⁹² Matrigel or LN-332 coated surfaces induced elevated surfactant protein C gene expression in differentiating mES cells, which suggests a direct indication of lung epithelial induction. The selection of the ECM protein coating on cultivation plates can greatly affect the induction of ES cells as well as MS cells. In particular, LN-332-coated PDLLA provides an ECM-degradable scaffold in combination with defined biomaterials, which should be appropriate for regenerative medicine of lung tissue construct.

2.6 Fibronectin

FN is a glycoprotein with a high molecular weight (~ 440 kDa), which binds to integrins and to ECM components such as heparan sulfate proteoglycans (e.g., syndecans), fibrin, and COL.^{198,199} FN presents as a protein dimer, composed of two almost identical molecules connected by a pair of disulfide bonds¹⁹⁸ and is known to play a significant part in cell differentiation, migration, growth, and attachment. Its RGD (Arg–Gly–Asp) sequence is the domain of cell adhesion through $\alpha V\beta 3$ and $\alpha 5\beta 1$ integrins. FN also has a cell attachment site of the connecting segment-1 (EILDVPST, CS1) that is typically identified by hematopoietic stem and progenitor cells. Table 2.7 lists some studies of FN-coated plates and the FN scaffolds used for MS cell differentiation as noted in the articles.^{25,41,45,51,52,56,62,67,70,71,75,76,110,183,196,200–203}

The attachment of hADS cells to FN is considered to be mediated by heparin-binding domain and $\beta 1$ integrin, which is verified from inhibitory measurements using heparin-binding peptide and an antibody against $\beta 1$ integrin, whereas the attachment of LN and COL seems to be definitely mediated by $\beta 1$ integrin.⁴⁵ $\beta 1$ integrin is a common receptor on MS cells, which mediates cell attachment to LN, FN, and COL type IV and type I.

Heparan sulfate proteoglycans are responsible for cell attachment of MS cells through the heparin-binding site of FN, and heparan sulfate proteoglycans regulate the osteogenic induction of MS cells through bone morphogenetic protein pathways.^{204,205} hADS cells cultivated on FN-coated plates induced into adipocytes to a greater extent than cells cultivated on TCP plates.²⁰⁶ However, hADS cells cultivated on FN-coated dishes induced into adipocytes less than hADS cells on the heparin-binding domain surface,²⁰⁶ because the cells retained much rounder morphologies when cultivated on a heparin-binding domain surface than on TCP plates and FN-coated plates. Furthermore, it has been demonstrated that hMS cells induced into osteoblasts under cultivation conditions that kept spread shapes, whereas rounded cells differentiated into adipocytes.²⁰⁶

Li and his colleagues attempted to enhance neuronal differentiation of rat neural stem/progenitor/stem (NSP) cells by the combination of appropriate biomaterials and ECMs (LN and FN).¹⁹⁶ The biomaterials selected in this study were polyvinyl alcohol (PVA), poly(ethylene-co-vinyl alcohol) (EVAL) and polyvinylidene fluoride (PVDF). The purpose of this study was to induce the differentiation of NSPCs more towards neurons than glial cells by the combination of media, biomaterials and ECMs.

The NSPCs were cultured in serum-containing medium for 7 days and it was demonstrated that the molecules being present in serum with molecular weight <100 kDa promoted neuronal differentiation of NSP cells, which could dominate the differentiation of NSP cells principally into neurons.¹⁹⁶ Furthermore, NSP cells were induced to differentiate predominantly into glial cell phenotypes on any biomaterials coated with and without ECMs in the presence of whole serum components. MAP2-positive (neuron)

Table 2.7 Investigations into stem cell induction on 2D and 3D fibronectin bio-materials.¹ Reproduced from ref. 1 with permission from the American Chemical Society, Copyright 2012.

Stem cell source ^a	Material for stem cell culture ^b	Differentiation	Reference
hBMSCs	Fibronectin/CP/HAP (2D culture, coating on HAP)	Osteoblasts	200
hBMSCs	Fibronectin (2D culture, coating on dishes)	Osteoblasts	25
hBMSCs	Fibronectin (2D culture, coating on dishes)	Osteoblasts	25, 62, 67, 110
hBMSCs	Fibronectin (2D culture, coating on dishes)	Osteoblasts, adipocytes	71
hBMSCs	Fibronectin (2D culture, coating on PDMS disk)	Osteoblasts, adipocytes	203
mBMSCs	Fibronectin (2D culture, coating on dishes)	Osteoblasts, adipocytes	70
mBMSCs	Fibronectin (3D culture, conjugating on PEG hydrogels)	Osteoblasts	195
hADSCs	Fibronectin (2D culture, coating on dishes)	Adipocytes	45
hADSCs	Fibronectin (2D culture, coating on dishes)	Cardiomyocytes	183
mESCs	Fibronectin (3D culture, coating on gelatin/PCL nanofibers)	Cardiac progenitors	161
hBMSCs	Fibronectin (2D culture, coating on dishes)	Smooth muscle cells	52
hBMSCs	Fibronectin (2D culture, coating on dishes)	Neural cells	56
hESCs (TE03, TE06)	Fibronectin/PDL (2D culture, coating on dishes)	Neural cells	51
Rat neural stem cells	Fibronectin (2D culture, coating on dishes)	Neurons	196
Human neural stem cells	Fibronectin (2D culture, coating on dishes)	Oligogliocytes	41
mESCs	Fibronectin (2D culture, coating on dishes)	Lung epithelium	192
BMSCs	Fibronectin (2D culture, coating on dishes)	Hepatocytes	201
Mouse hepatic stem cells	Fibronectin (2D culture, coating on dishes)	Hepatocytes	75
hESCs (H9)	Fibronectin/PLGA + PLLA (3D culture, scaffold)	Endoderm cells, ectoderm cells, chondrocytes	202

^aADSCs, adipose-derived stem cells; BMSCs, bone marrow stromal cells; ESCs, embryonic stem cells; hADSCs, human ADSCs; hBMSCs, human BMSCs; mBMSCs, murine BMSCs; hESCs, human ESCs; mESCs, murine ESCs.

^bCP, calcium phosphate; HAP, hydroxyapatite; PCL, polycaprolactone; PDL, poly-D-lysine; PDMS, polydimethylsiloxane; PLGA, poly(lactic-co-glycolic acid); PLLA, poly-L-lactic acid.

percentage of the migrated cells could be promoted over 85% on FN-coating EVAL biomaterials based on the medium containing serum fraction.¹⁹⁶ These results, which are favorable for neuronal differentiation, should be useful in the development of strategies for controlling the behavior of NSP cells in neuroscience investigation.

Chang and his colleagues examined that a pellet suspension cultivation of hMS cells with the addition of FN facilitated induction of MS cells to pancreatic, insulin-producing cells, with elevated Glut2 and insulin gene expression.²⁰⁷ A four-stage protocol that includes neuronal induction factor and insulin-producing cell (IPC)-conversion reagent (nicotinamide) is typically used to derive IPCs from ES cells, but was shown not to be sufficient to induce MS cells to undergo IPC induction in 2D cultivation.²⁰⁷ On the other hand, the pellet suspension cultivation of hMS cells with the addition of FN promoted pancreatic induction. The differentiated cells could secrete insulin in reaction to increased glucose concentration, and this was controlled by reagents that enhanced cyclic AMP generation and adjusted Ca influx.²⁰⁷ LN-1 facilitated the induction of fetal mouse pancreatic β -cells.^{207,208} More studies of the mechanisms by which ECM proteins regulate the facilitation of IPC differentiation are necessary.

Sogo and his colleagues made HYA ceramic composites immobilized with COL type I and/or FN.²⁰⁰ The calcium phosphate precipitate and ECM proteins generated a composite surface layer, and ECM proteins were released partially for 72 h into a physiological salt solution.²⁰⁰ hMS cells cultivated on the HYA ceramic composite having FN produced higher ALP activity in osteogenic induction media than hMS cells on the HYA ceramic composite having COL type I, which suggests that hMS cells induced into osteogenic lineages on the HYA ceramic composite having only FN.²⁰⁰ There was no synergetic effect of hMS cell differentiation into osteoblasts on the HYA ceramic composite having both COL type I and FN. Therefore, the FN-HYA composites but not the COL type I-HYA composites are preferable for the facilitation of osteogenic induction of hMS cells *in vitro*.

2.7 Vitronectin

Vitronectin, which is an ECM glycoprotein, participates in the differentiation of diverse cell types in adult and embryonic tissues.^{209,210} Vitronectin is not typically used for coating or scaffold materials except coating dishes for hES and hiPS cell culture, although vitronectin exists abundantly in serum. A few studies have described the positive effects of vitronectin on differentiation of MS cells in scaffolds, hydrogels, and 2D cultivation.^{25,62,71,209}

Vitronectin could facilitate the formation of spinal motor neurons by synergistically regulating with sonic hedgehog (Shh), both in explants and neuroepithelial cell cultivation of chick embryo spinal cord.^{209,211} Motor neurons and oligodendrocytes could be taken from a common pool of spinal

cord progenitors.^{212,213} Vitronectin is an appropriate candidate to facilitate the induction of motor neurons as well as spinal cord oligodendrocytes.

Gil and his colleagues observed that the oligodendrocytic induction of hES cells was sufficiently facilitated by vitronectin.²⁰⁹ Salaszyk studied osteogenic induction of hMS cells cultivated on plates coated with LN-1, vitronectin, COL type IV, COL type I, and FN.²⁵ hMS cells were found to attach ECM proteins in this order: LN-1 ≤ vitronectin ≤ COL type IV ≤ COL type I ≤ FN. The cells cultivated on plates coated with COL type I and vitronectin induced into osteoblasts to a higher extent than cells on plates coated with LN-1 or FN, as evaluated by mineral deposition, osteopontin expression, and ALP activity.²⁵ The interaction of hMS cells with COL type I and vitronectin seems to facilitate the osteogenic induction of hMS cells.

The geometry of the matrices (*i.e.*, 3D *versus* 2D) plays a major role in deciding how a cell reacts to biochemical and/or mechanical cues, because most native cells in tissues are surrounded by ECMs.²¹⁴ Therefore, Heydarkhan-Hagvall examined a 3D microenvironment using electrospun fibers made of gelatin and PCL and investigated the impact of several ECM proteins and geometry on the development of cardiac progenitor (Flk-1⁺) cells from murine ES cells and their differentiation into functional cardiovascular cells.¹⁶¹ They studied the effect of COL IV, FN, LN and vitronectin on the adhesion and proliferation of murine ES cells and further investigated the effects of ECM proteins on the number of Flk-1⁺ cells cultivated in 2D conditions compared to a 3D culture system in a feeder-free condition.¹⁶¹ The results indicated that the number of Flk-1⁺ cells was significantly higher in 3D nanofiber scaffolds coated with LN or vitronectin compared to COL IV-coated nanofiber scaffolds (Figure 2.10).¹⁶¹ This study shows the importance of defined culture systems *in vitro* for evaluation of the guided differentiation of ES cells in the field of cardiovascular tissue engineering.

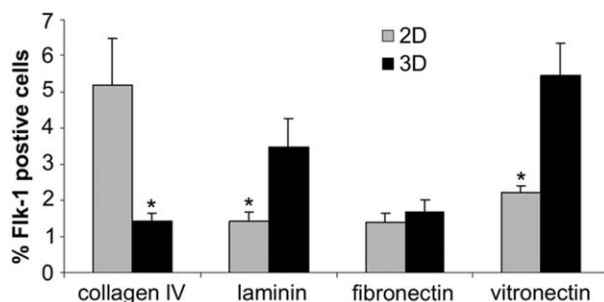


Figure 2.10 FACS assay of differentiating mES cells cultivated on COL type IV, vitronectin, LN and FN for expressing the number of Flk-1⁺ cells in 2D and 3D cultivation system after 4 days. The number of Flk-1⁺ cells was much higher in 2D COL type IV-coated dishes. However, the number of Flk-1⁺ cells in 3D vitronectin-coated scaffolds was much higher than in 3D COL type IV-, LN- and FN-coated scaffolds.¹⁶¹ Adapted from ref. 161 with permission from Elsevier, Copyright 2012.

2.8 Fibrin

Many cells such as human neural stem/progenitor (hNSP) cells die after implantation into defective sites in patients. This cell death can be avoided to some extent by the use of biomaterial scaffolds, if optimal scaffolds are identified for hNSP cell entrapment and transplantation. Therefore, Arulmoli and his colleagues examined the characteristics of fibrin-based scaffolds and their effects on hNSP cell proliferation and differentiation.¹⁹³

Fibrin is an ECM, which is responsible for blood clotting during the coagulation cascade and is biocompatible and non-toxic. Fibrin hydrogels are generated when fibrinogen is cleaved by thrombin to make fibrin monomers, which are covalently crosslinked by Factor XIIIa to generate a mesh, and the fibrin hydrogels can be degraded by the plasmin enzyme. The mechanical properties and polymerization time of the fibrin hydrogel can be adjusted by varying the concentrations of fibrinogen and thrombin.^{193,215} Fibrin has multiple adhesive sites such as RGD sequences, which interact with integrin receptors on the cell surface. Fibrin hydrogels were used as scaffolds for NSP cells as well as a vehicle for growth factor delivery in some models of spinal cord injury.^{216–219}

Salmon fibrins fit to the mechanical properties of tissues in CNS.^{220,221} Higher locomotor functional improvement, density of serotonergic fibers caudal to the lesion site, and improvement of bladder function over mammalian fibrin were reported when salmon fibrins were used to treat rats with injuries of the dorsal hemisection spinal cord.²²² Although salmon fibrins are found to be an effective scaffold to treat CNS injury,²²² salmon fibrins degrade very fast *in vivo* (a few days) and thus are unlikely to provide long-term support for implanted hNSP cells.¹⁹³

Arulmoli and his colleagues found the fibrin scaffolds composed of salmon fibrinogen and thrombin stimulated higher proliferation of hNSP cells than those made of mammalian fibrin.¹⁹³ However, fibrin scaffolds degraded much too fast, just a few days *in vivo*. Therefore, they intended to develop novel scaffolds, which have the beneficial characteristics of fibrins but degrade much slower than conventional fibrin scaffolds, which support longer periods of hNSP cell survival. The results suggested that the scaffolds made of salmon fibrins with IPNs (interpenetrating networks) of HA without and with LN were found to be compliant hydrogels, which match the physical properties of brain tissue.¹⁹³ Furthermore, fibrin scaffolds with HA and LN supported hNSP cell proliferation significantly and induced differentiation of hNSP cells while the composited fibrin scaffolds significantly decreased the cell-mediated degradation, which was generally observed in the fibrin scaffolds solely made of fibrin alone.¹⁹³ The hNSP cells expressed two fibrinogen-binding integrins, $\alpha_v\beta_1$ and $\alpha_5\beta_1$, as well as several LN binding integrins ($\alpha_7\beta_1$, $\alpha_6\beta_1$, $\alpha_3\beta_1$), which can mediate interaction with the scaffold. They also reported enhanced vessel formation to evaluate the ability of scaffolds by co-culture of human cord blood-derived endothelial

cells and hNSP cells in the fibrin scaffolds with HA and LN compared to the fibrin scaffolds made of fibrin only.¹⁹³

2.9 Decellularized ECM

The biological environment of cells *in vivo* regulates stem cell fate and controls MS cells to induce into specific lineages. It is rather hard to reproduce biological niches using only pure ECM proteins, glycosaminoglycans, and other components *in vitro*. One of the methods to mimic a biological environment *in vitro* is to use decellularized ECM.^{223–228} Decellularization is a good technique to remove cellular components from native tissues and is typically made by a combination of chemical, physical, or enzymatic methods.^{229,230} This technique rejects the xenogenic or allogenic cellular antigens, as well as cellular compositions, from the tissues, but reserves the ECM components.²³¹ Several researchers have focused on the decellularization of organs and tissues such as nerves, skin, blood vessels, lung, liver, heart, and heart valves.^{223–226} Decellularization is commonly done by surfactant or freeze–thaw cycling protocols.^{70,231–237} The freeze–thaw cycling protocol is as follows: the scaffold is thawed in a temperature-controlled bath at 37 °C for 10 min, washed with PBS (phosphate-buffered saline) to remove cellular debris, and frozen in liquid nitrogen for 10 min. Then, the scaffold is left at room temperature for 1 hour to melt. Subsequently, the scaffold undergoes several freeze–thaw cycles under sterile conditions to ensure the complete removal of the cellular compositions. After immersion in NH₄OH aqueous solution and washing with PBS, the scaffold is dried under air before being inoculated with cells.^{233,238}

The typical surfactant protocol is as following processes. Cells were treated with 0.1% Triton X-100 in water at room temperature for half hour. Cell lysates were meticulously aspirated, and a solution of concentrated NH₄OH diluted 1 : 100 in water was gently added to the wells for 5–7 min. The wells were rinsed twice with PBS and stored or used rapidly in PBS at 4 °C.^{236,239} Acellular ECMs prepared from xenogenic or allogenic tissues have been used as scaffold for the tissue engineering of ligaments,^{231,240} tendons, nerves,²⁴¹ vessels,²⁴² and heart valves.^{243,244} Several landmark works of MS cell differentiation and expansion that are investigated by cultivation on decellularized ECMs are shown in Table 2.8.^{70,227,228,231–237,245–248}

Several researchers have indicated that ECM regulates glial growth and neuritogenesis.^{236,249,250} However, little is known about the effect of MS cell-derived ECMs on neural cells. Aizman and his colleagues described that the ECMs generated by MS cells could maintain neural cell growth and attachment *in vitro*. They examined the neurosupportive characteristics of MS cells to MS cell-derived SB623 cells that were being prepared as a cell therapy for stroke.²³⁶ Embryonic rat brain cortical cells cultivated for 3 weeks on SB623 cell-derived ECMs and hMS cell-derived ECM showed about 3- and 1.5-fold higher metabolic activities, respectively, in comparison to cultivation grown on PDL-coated plates.²³⁶ The SB623-derived ECMs and MS cell-derived ECMs

Table 2.8 Investigations into stem cell induction on decellularized extracellular matrix biomaterials.¹ Reproduced from ref. 1 with permission from the American Chemical Society, Copyright 2012.^a

Stem cell source	Material for stem cell culture	Differentiation	Ref.
mBMSCs	Decellularized ECM from mBMSCs (2D culture)	Pluripotency, osteoblasts, adipocytes	70
mESCs (E14 TG2a)	ECM from decellularized osteoblasts and non-osteogenic cells (2D culture)	Osteoblasts	235
Rat BMSCs	Decellularized ECM on electrospinning fibers of poly(ϵ -caprolactone) from osteoblasts differentiated from rat BMSCs	Osteoblasts	246
Rat BMSCs	Decellularized ECM from osteoblasts differentiated from rat BMSCs on titanium fiber mesh (3D culture)	Osteoblasts	233
hBMSCs	Decellularized bovine endosteum-derived particles (3D culture)	Osteoblasts, chondrocytes, adipocytes	232
hAMSCs	Human placenta-derived decellularized ECM sponges (3D)	Osteoblasts, chondrocytes	228
Rabbit BMSCs	Decellularized ECM scaffold from porcine cartilage (3D culture)	Chondrocytes	245
hBMSCs	Decellularized ECM from chondrocyte-encapsulated collagen microspheres (3D culture)	Chondrocytes	231
hADSCs	Porous scaffold derived from decellularized articular cartilage (3D culture)	Chondrocytes	237
hBMSCs	Decellularized scaffolds on PLGA, which are derived from hBMSCs and chondrocytes	Chondrocytes	238, 252
hBMSCs	Gelatin hydrogels with soluble decellularized ECM from bovine meniscus	Chondrocytes	247
Rat BMSCs	Decellularized ECM particles from goat cartilage (3D culture)	Chondrocytes	248
mESCs (D3)	Decellularized ECM scaffolds from mESCs, which are crosslinked	Neural cells	227
Embryonic rat brain cortical cells	Decellularized ECM from hBMSCs (2D culture)	Neural cells	236
Human urine-derived stem cells	Decellularized small intestinal submucosa scaffold (3D culture)	Urethral tissue composed of urothelial and smooth muscle cells	234

^aADSCs, adipose-derived stem cells; BMSCs, bone marrow stromal cells; ECM, extracellular matrix; ESCs, embryonic stem cells; hADSCs, human ADSCs; hAMSCs, human amniotic membrane-derived stem cells; hBMSCs, human BMSCs; mBMSCs, murine BMSCs; mESCs, murine ESCs.

protected neural cells from growth factor and nutrient deprivation, and helped the growth of oligodendrocytes, astrocytes, and neurons.²³⁶ Morphologically, neurons on cell-derived ECMs generated more extended and complex neurite networks than the neurons cultivated on PDL-coated plates. It was indicated that the cell-derived ECMs would be a regulator of the neuroregenerative characteristics of the SB623 cells and MS cells *in vivo*.²³⁶

Pluripotent stem cells (PSCs) and their progeny secrete a significant amount of ECMs at various developmental stages, which could interact with regulatory growth factors to regulate differentiation commitment of stem cells. ECMs derived from PSCs can be used as unique scaffolds that provide broad signaling abilities to mediate cellular differentiation. However, the rapid degradation of ECMs is a barrier to their application as the scaffolds for transplantation *in vivo* as well as cell expansion and differentiation *in vitro*. Therefore, Sart and his colleagues studied the effects of crosslinking on the ECMs derived from murine ES cells and the regulatory capacity of the crosslinked ECMs on the proliferation and differentiation of ES cell-derived NPCs (neural progenitor cells).²²⁷ The undifferentiated aggregates, spontaneous EBs, and ES cell-derived NPC aggregates were decellularized to generate different biological cues. The derived ECMs were crosslinked using glutaraldehyde (GA) or genipin to enhance the scaffold stability. The genipin, which is extracted from the fruit of the *Gardenia jasminoides Ellis* plant, has low toxicity and is considered to be a natural crosslinking agent.¹⁰⁷

ES cell-derived NPC aggregates were seeded on different crosslinked ECM scaffolds and differential cellular compositions of neural progenitors, neurons, and glial cells were observed, which were dependent on different ECM scaffolds. Figure 2.11 shows expression of neuronal marker (β III tubulin) and astrocyte marker (GFAP) of NPC aggregates on decellularized ECM scaffolds, which were crosslinked with genipin (DE-NG) and not crosslinked (DE-NC).²²⁷ NPC aggregates were found to enhance neuronal differentiation and reduce astrocyte differentiation on crosslinked DE-NG scaffolds compared to those on non-crosslinked DE-NC scaffolds. Crosslinking of decellularized ECM scaffolds modulates the structural and biophysical properties of the ECM scaffolds, which enrich the neuronal cell population over the glial (astrocyte) cell population.²²⁷

ES cell-derived ECM scaffolds were found to affect neural differentiation through intrinsic biological cues and biophysical properties. These scaffolds may have potential for tissue regeneration *in vivo* as well as cell culture and differentiation *in vitro* studies.

Cheng and his colleagues studied whether scaffolds obtained from articular cartilage would induce chondrogenesis of hADS cells.²³⁷ hADS cells were seeded on porous scaffolds derived from adult porcine articular cartilage and cultured in standard medium without exogenous growth factors. Chondrogenesis of hADS cells seeded within the scaffold was shown by quantitative RT-PCR assay of cartilage-specific ECM genes (aggrecan and COL type II).²³⁷ Histological evaluation indicated high generation of cartilage-specific ECMs (COL type II) after 4–6 weeks of cultivation.

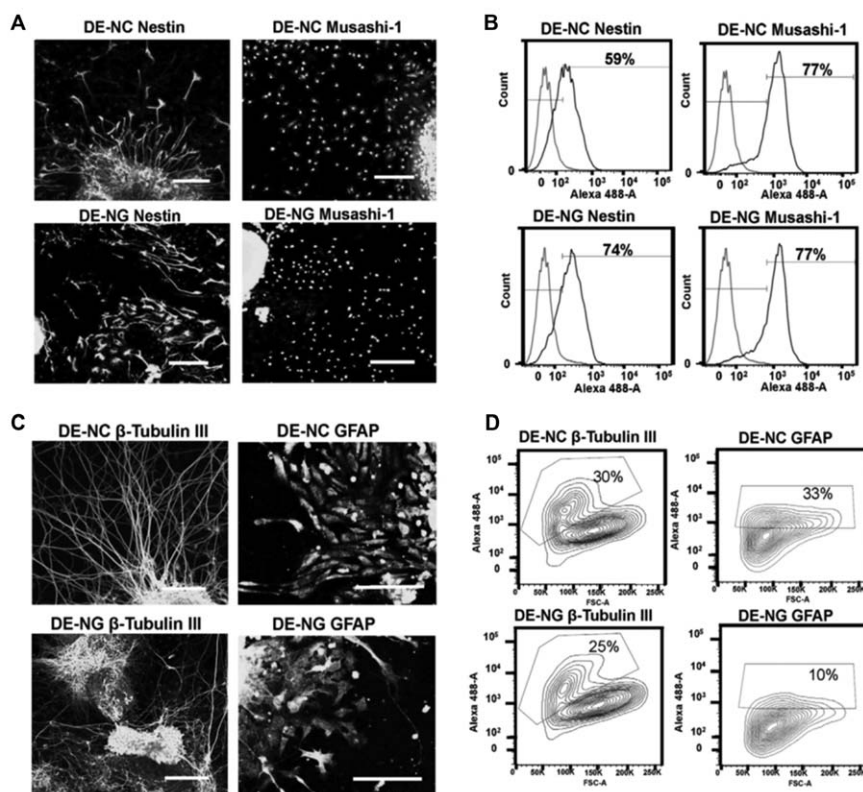


Figure 2.11 Expression of neural markers of the ES cell-derived NPCs grown on decellularized ECM scaffolds, which were crosslinked with genipin (DE-NG) and not crosslinked (DE-NC). (A) Representative confocal fluorescent images of Nestin and Musashi-1 expression. Scale bar: 100 μ m. (B) Representative flow cytometry histograms of Nestin and Musashi-1 expression. (C) Representative confocal fluorescent images of β -tubulin III and GFAP expression. Scale bar: 100 μ m. (D) Representative flow cytometry histograms of β -tubulin III and GFAP expression.²²⁷ Adapted from ref. 227 with permission from Elsevier, Copyright 2016.

The morphologies of cells in the hADS cell-inoculated scaffolds were similar to the morphologies of natural articular cartilage tissues, with rounded cells locating in the glycosaminoglycan-rich domains of the scaffold after 6 weeks of cultivation.²³⁷ Biphasic mechanical evaluation indicated that the aggregate modulus of the hADS cell-inoculated scaffolds increased over time, reaching 150 kPa by day 42, more than three-fold higher than that of the unseeded controls.²³⁷ These findings indicate that porous scaffolds prepared from articular cartilages have the potential to induce differentiation of hADS cells into chondrocytes with no exogenous growth factors, which lead to accumulation and synthesis of ECMs and the development of mechanical properties similar to those of natural cartilage.²³⁷ These results support the

abilities of processed cartilage ECMs as biopolymer scaffolds for cartilage regenerative medicine.²³⁷

Osteochondral tissue engineering is valuable for clinical aspects of the treatment and management of cartilage and underlying bone. Therefore, Rameshbabu and his colleagues prepared human placenta-derived ECM scaffolds (hPECMs) for treatment of osteochondral tissues using a decellularization process.²²⁸ They found that no significant cellular components existed in the hPECMs. In particular, there was no significant alteration for the COL and glycosaminoglycan (native ECMs) content of the hPECMs. hPECMs *in vitro* presented a non-cytotoxic environment, which is rich in bioactive cues for hAMS cells (human amniotic membrane-derived stem cells) to proliferate in and differentiate into chondrogenic and osteogenic lineages under induction.²²⁸ When the hPECMs were subcutaneously implanted into rabbits, histological analysis at 28 days demonstrated no severe immune response in the host and supported the formation of blood vessels. The cell-free hPECMs (CFP) and cell-seeded hPECMs (CSP) were implanted at osteochondral defect sites in a rabbit to assess the osteochondral tissue repair ability of hPECMs (Figure 2.12).²²⁸ Histological analysis indicated that osteochondral regeneration was more successful in the defects filled with CSP compared to those filled with CFP and empty defects after 60 days of implantation.²²⁸ The results indicate that a naturally derived biocompatible scaffold composed of ECMs from human placenta was successfully developed for osteochondral tissue engineering.

Evans and his colleagues examined whether tissue-specific ECMs affected the induction of ES cells.²³⁵ They induced murine ES cells to differentiate by EB generation, following dissociation and cultivation of EBs on ECM made by decellularization of either non-osteogenic cell (A549) or osteogenic cell (MC3T3-E1) cultivation, or on defined COL type I matrices.²³⁵ The osteogenic induction was examined by osteogenic gene expression and generation of mineralized tissue, and was much higher on ECM matrices prepared from

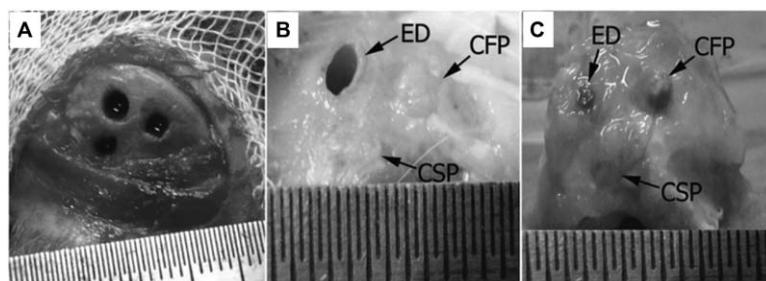


Figure 2.12 Photomicrograph of the drilled osteochondral defect sites (A) treated with (B) CFP (cell-free hPECMs [human placenta-derived ECM scaffolds]) and CSP (cell-seeded hPECMs) and (C) complete joints, which were analyzed after 60 days.²²⁸ Adapted from ref. 228 with permission from the Royal Society of Chemistry.

MC3T3-E1 cells than on any other ECM matrices. The osteogenic effect of the MC3T3-E1 matrices was decreased by thermal treatment and stopped by trypsin treatment, indicating that bioactive molecules produced by MC3T3-E1 cells were the important factors, which facilitated induction of ES cells into the osteogenic lineage.²³⁵ These findings show that decellularized, bone-specific ECMs can facilitate the osteogenic induction of ES cells with incorporation of tissue-specific ECM signals, which stimulate stem-cell differentiation.

Datta and his colleagues studied the effect of ECMs laid down by osteoblastic cells on the osteoblastic induction of rat BMS cells.²³³ Primary rat BMS cells inoculated in titanium (Ti) fiber scaffolds were induced into osteoblasts in static cultivation, and then the scaffolds were decellularized by fast freeze–thaw cycling. Decellularized scaffolds were re-seeded with rat BMS cells, and osteogenicity was examined using osteopontin, calcium, ALP, and DNA assay. Calcium was deposited at a higher rate by cells cultured on decellularized scaffolds than control scaffolds by 16 days.²³³ The Ti/BMS cell constructs indicated negligible calcium content at 16 days, compared with 210 mg/construct for the Ti/ECM/MS cell constructs cultivated with no osteogenic supplements.²³³ These findings suggest that bone-like ECMs synthesized *in vitro* can facilitate the osteoblastic induction of MS cells.

Wu and his colleagues manufactured urethral tissue engineered from UCs (urothelial cells) prepared from USCs (urine-derived stem cells) that were inoculated on 3D porous scaffolds made by decellularization of SIS (pig small intestinal submucosa).²³⁴ Differentiated SMCs and UCs were inoculated onto the SIS scaffold in a layered co-cultivation process and cultivated for 7 days. The seeded cells generated multiple uniform layers on the SIS and migrated deeper into the porous matrices.²³⁴ USCs, which were induced to differentiate, showed expression of SM cell markers (myosin, desmin, and α -SM actin) or UC markers (AE1/AE3 and Uroplakin-III) after transplantation into athymic mice for 30 days.²³⁴ Thus, SM cells and UCs prepared from USCs could be kept on 3D porous SIS scaffolds. The dynamic cultivation system further facilitated 3D cell matrices in-growth and development of a multilayer mucosal morphology, which is similar to natural urinary tract tissues.²³⁴ USCs might be used as an alternative cell source for cell-based regenerative medicine for urological tissue repair and urethral reconstruction.

Depending on the cells from which decellularized ECMs are taken, the ECMs can not only facilitate appropriate induction lineages of MS cells but can also inhibit MS cell induction. Chen and his colleagues found that ECMs secreted by murine BMS cells promoted the proliferation of MS cells and inhibited their induction into osteoblastic cells.⁷⁰ The differentiation potential of MS cells gradually decreased with extensive passaging when MS cells were cultivated on TCP dishes.²⁵¹ This is explained by the fact that BM niches, which promote retention of stem cell characteristics, are missing in TCP dish cultivation.⁷⁰ Therefore, the potential of BMS cell-derived ECMs to maintain the stemness of MS cells *in vitro* was investigated. The BMS cell-derived ECMs consisted of decorin, biglycan, LN, FN, perlecan, syndecan-1,

and COL types I, III, and V, similar to the components of the marrow ECMs.⁷⁰ These ECMs facilitated MCFU (mesenchymal colony-forming unit) replication, suppressed their “spontaneous” induction toward the osteoblast lineage, and kept their potential to induce into adipocytes or osteoblasts.⁷⁰ The implantation of MCFUs proliferated on the BMS cell-derived ECMs into immunocompromised mice created eight times more hematopoietic marrow and five times more bone than MCFUs cultivated in TCP plates.⁷⁰ According to this investigation, ECMs in BMS cells play a key role in supporting MS cell stemness.

Lu and his colleagues manufactured ECM scaffolds derived from chondrocytes and MS cells on PLGA mesh.²³⁸ ECM-PLGA-cell matrices were decellularized by a freeze-thaw method, and then dipped into aqueous Na_3PO_4 solution to delete the PLGA mesh templates. The decellularized ECM biomaterials were found to retain a higher stimulatory effect on chondrogenic induction of MS cells in comparison to typical pellet cultivation.²³⁸ In particular, decellularized ECM scaffolds made from MS cells exhibited higher promotion of MS cells into chondrogenic differentiation than did ECM scaffolds made from chondrocytes.²³⁸ This preparation protocol opens up a route for efficient generation of autologous ECM (aECM) scaffolds by decellularizing the resulting cell-ECM constructs and cultivation of autologous cells.^{238,252} The use of patient BMS cells and ECM scaffolds is expected to induce the desired responses for clinical therapy.^{238,252–255}

2.10 Biomaterials with ECM-mimicking Oligopeptides

We have seen that MS cells on scaffolds or hydrogels with immobilized ECM proteins or plates coated with ECM proteins are able to efficiently facilitate the induction of MS cells into specific lineages. However, several technical challenges exist. It is not possible to preserve the dishes, scaffolds, and hydrogels including ECMs at room temperature, and so these biomaterials including ECMs must be kept in a refrigerator under sterile conditions. Moreover, it is not easy to sterilize dishes, scaffolds, and hydrogels containing ECMs because denaturation of ECMs must be avoided when immobilized ECMs are used in clinical treatments. The inclusion of cell adhesive peptides from ECMs, which are extremely stable and have lower molecular weights than ECMs, in the design of scaffolds, hydrogels, and coating biomaterials on plates, is an extremely valuable strategy. ECM-derived oligopeptides (ECM-peptides) can be grafted or coated onto cell cultivation plates for 2D cultivation of MS cells,^{256–258} and ECM-peptides would be non-covalently or covalently included in hydrogel or scaffold networks for 3D cultivation.^{24,42,259–270} Moreover, ECM-peptides can make nanofiber configurations by self-assembly.^{22,271,272}

Table 2.2 summarizes some cell-binding domains of ECMs, together with original ECMs from which they are derived and the attachment sites of

integrins. Oligopeptides of IKVAV, YIGSR, DGEA (binding to $\alpha 2\beta 1$ integrin), and RGD (binding to $\alpha 5\beta 1$ integrin or VLA-5) are currently used for this purpose. Table 2.9 shows several examples of research on MS cell differentiation and cultivation in scaffolds or hydrogels with immobilized ECM-peptides or on plates coated (or grafted) with ECM-peptides.^{22,24,260–266,268–299}

Santiago and his colleagues designed the PCL surfaces covalently conjugated with IKVAV, YIGSR, and RGD oligopeptide obtained from LN and examined the adhesion and expansion of ADS cells.²⁶⁹ IKVAV-treated biomaterials showed a much higher number of bound ADS cells at 2 and 3 days after cell inoculation in comparison to other oligopeptide sequences.²⁶⁹ Their findings suggested that IKVAV seems to be an adequate oligopeptide sequence for use in surface modification aimed at enhancing the adhesion of ADS cells to tissue-engineered scaffolds.²⁶⁹ On the other hand, several other researchers have shown that other ECM-oligopeptides were as or more effective for stem cell adhesion on plates and scaffolds, which depend on the base biomaterials of plates and scaffolds.^{24,260–264,271}

The effect of ECM-peptides in the plates, scaffolds, and hydrogels immobilized ECM-oligopeptides on potential of MS cells into optimal lineages is described in the following sections.

2.10.1 MS Cell Differentiation on Self-assembled ECM-peptide Nanofibers

Self-assembled nanostructure in scaffolds is especially important because the self-assembled structure mimics the hierarchical structure and self-assembled generation of natural tissues. Oligopeptide amphiphile (PA) is reported to spontaneously form self-assembled nanofibers above the critical micelle concentration.^{22,271,272} Anderson and his colleagues made oligopeptide amphiphile nanofibers grafted with appropriate cellular adhesion ligands (*i.e.*, KRSR, DGEA, and RGDS) and studied whether the nanofibers could guide osteogenic induction of hMS cells with no osteogenic supplements.²⁷¹ The oligopeptide amphiphile nanofibers existed as self-assembled 2D coatings on the plates. hBMS cells cultivated on the RGDS-containing oligopeptide amphiphile nanofibers, but neither KRSR nor DGEA nanofibers, exhibited much higher ALP activity, suggesting the early promotion of osteogenic induction, and exhibited a progressive shift into osteogenic morphologies and a mineral deposition.²⁷¹ The oligopeptide amphiphile nanofibers that mimic the natural ECMs in bone were observed to guide the osteogenic induction of hBMS cells without the aid of supplements to some extent, and presented an adaptable environment that allowed several adhesion sites to regulate cellular behaviors.²⁷¹

The self-assembly peptide forms hydrogels, which are valuable biomaterials for regenerative medicine because the formation of the hydrogels is generated by spontaneous self-assembly of oligopeptides without

Table 2.9 Investigations into stem cell induction on ECM-peptide biomaterials.¹ Reproduced from ref. 1 with permission from the American Chemical Society, Copyright 2012.^a

Stem cell source	Material for stem cell culture containing ECM peptide	Differentiation	Ref.
hBMSCs	ECM-mimicking peptide (RGDS, DGEA, KRSR) amphiphile nanofiber (2D culture, coating on dishes)	Osteoblasts	271
hBMSCs	Silk scaffold bound GRGDS covalently (3D culture, scaffold)	Osteoblasts	266
hBMSCs	Collagen mimetic peptide (DGEA, P15 (GTPGPQIAGQAGVV), QAGVV, GFOGER) and GPenGRGDSPCA (3D culture, coating on HYA)	Osteoblasts	24
hBMSCs	PEG hydrogels grafted with $\alpha 5$ integrin binding peptide (cyclic RRETAWA)	Osteoblasts	276
hBMSCs	Alginate hydrogels grafted with osteoinductive oligopeptide (GGGYGFGG, GGPVGLIYGFGG, GGGIVGOLGYGFGG)	Osteoblasts	277
hBMSCs	Self-assembled peptide (FEFEFKFK) nanofiber hydrogels	Osteoblasts	278
hBMSCs	Collagen type I-based recombinant peptide scaffolds	Osteoblasts	279
gBMSCs	PEODA (polyethylene glycol diacrylate) incorporated with YRGDS (3D culture, gel)	Osteoblasts	260
mBMSCs	BMP-2 mimicking peptide (CGKIPKASSVPTLSAISTLYL)-grafted alginate hydrogels	Osteoblasts	280
mBMSCs	Alginate hydrogels grafted with oligopeptide (GGGGDGEASP, GGGGRGDSP)	Osteoblasts	281
hiPSCs	Carboxymethylchitosan hydrogels grafted with vitronectin-derived oligopeptides (KGGPQVTRGDVFTMP)	Osteoblasts	282
Rat dental pulp stem cells	Self-assembling peptide nanofiber hydrogels (SPG-178-Gel, RLDRLALRLDLR)	Osteoblasts	283
Rat BMSCs	RGD peptides (2D culture, grafting on PEG gel)	Osteoblasts, adipocytes	273
hBMSCs	Bioprinted PEG-Peptide (acrylated RGD, acrylated MMP) scaffold	Osteoblasts, chondrocytes	284
hBMSCs	Self-assembling peptide (KFE-9) (Ac-FKFEFKFE-NH ₂ and Ac-GRGDSP-GG-FKFEFKFE-NH ₂) nanofiber hydrogels	Adipocyte, chondrocytes, osteoblasts	285
hADSCs	Polysulfobetaine methacrylate hydrogels conjugated with angiogenic peptide (VEGF mimicking peptide) and RGD peptide	Adipocytes/ endothelial cells	286

Dental pulp stem cells	Self-assembled peptide (PuraMatrix) nanofiber hydrogels	Osteodentin	305
hBMSCs	PEG hydrogel containing ECM peptide motif (CRGDSC, CPENFFGGRGDSC) (3D culture, gel)	Chondrocytes	264
hBMSCs	PEG hydrogel containing RGDS (3D culture, gel)	Chondrocytes	262
hBMSCs	Elastin-like polypeptide (ELP, pentapeptide repeat [Val-Pro-Gly-Xaa-Gly]) hydrogel ^c (3D culture, gel)	Chondrocytes	268
hBMSCs	<i>N</i> -cadherin peptide containing PEG hydrogels (Ac-HAVDIGGKLDLKLKLDL)	Chondrocytes	300
hBMSCs	Self-assembled peptide (RAD16-I) nanofiber hydrogels	Chondrocytes	287
hBMSCs	Self-assembling peptide ((HSNGLPLGGGSEEEAAVVV(K)CO(CH ₂) ₁₀ CH ₃)) nanofiber hydrogels	Chondrocytes	303
hESC-derived MSCs	PEG hydrogel containing ECM peptide motif (YRGDS) (3D culture, gel)	Chondrocytes	263
gBMSCs	PEG hydrogel containing ECM peptide motif (collagen mimetic peptide ([Pro-Hyp-Gly] ₇ -Tyr) (3D culture, gel))	Chondrocytes	261
mBMSCs	PEG hydrogel containing matrix metalloproteinase-sensitive peptide (QPQGLAK) and chondroitin sulfate A (3D culture, gel)	Chondrocytes	265
Horse ADSCs and BMSCs	Self-assembling peptide (Ac-KLDLKLKLDL-NH ₂) nanofiber hydrogels	Chondrocytes	304
Rabbit ADSCs	Self-assembled peptide (Ac-(RADA)4-GPRGDSGYTGDS-NH ₂) nanofiber hydrogels	Chondrocytes	288
UC-MSCs	Self-assembled peptide (RADA16-BDNF) nanofiber hydrogels	Neural cells	289
Rat neural stem cells	Outer membrane protein A having ECM peptide motif (RGDS, GTPGPQGIAGQRGVV [collagen I], PHSRN [fibronectin], MNYYSNS [collagen IV], YIGSR [laminin]) (2D culture, coating on dishes)	Neural cells	22
Neural stem cells	Bacterial peptide (2D culture, coating on dishes)	Neural cells	274
Murine neural stem cells	Nanofiber scaffold of self-assembled peptide containing motif of laminin (YIGSR, IKVAV, PDSGR), collagen (DGEA, FPGERGVGPGP, PRGDSGYRGDS), fibronectin (RGDS), and bone marrow homing peptides (SKPPGTSS, PFSSTKT) (3D culture, scaffold)	Neural cells	272
mESCs	PEG hydrogels containing <i>N</i> -cadherin peptide (His-Ala-Val-Asp-Ile)	Neural cells	322
hPSC-derived NPCs	Vitronectin-derived peptide (CGKKQRFHRNRKG) (2D culture, coating on dishes)	Neuron	275

Table 2.9 (Continued)

Stem cell source	Material for stem cell culture containing ECM peptide	Differentiation	Ref.
NSCs	PEG hydrogels grafted with linear or cyclic RGD peptide	Neuron	290
hNSCs	Silk fibroin scaffold functionalized with IKVAV peptide	Neuron	291
hiPSC-derived neurons	Self-assembled peptide (RADA16-I ([RADA] ₄)) nanofiber hydrogels	Survival	292
hiPSC-CM	Peptide [oligopeptides (12 different RGD peptides)]-grafted PEG hydrogel microarrays	Adhesion	293
hBMSCs	PCL/gelatin nanofibrous scaffolds with collagen IV-derived peptide (KKGPRGDPPGF)	Proliferation	294
hBMSCs	EGF tethered tricalcium phosphate immobilized with EGF binding peptide	Survival of hBMSCs	295
hADSCs	RGD, YIGSR, and IKVAV grafted PCL (2D culture, disk)	Proliferation	269
UC-MSCs	Self-assembled thixotropic peptide (HO-WY-Suc-OH) bolaamphiphile hydrogels	Proliferation	296
Human synovium-derived MSCs	PCL electrospun mesh or decalcified bone scaffold grafted with peptide [L7 (LTHPRWP) and RGD peptide]	Adhesion and proliferation	297
Skin derived precursors and neonatal mouse epidermal cells	Self-assembling peptide [RADA-PRG (Ac-RADARADARADAGPRGDSGYRGDS-CONH ₂)] nanofiber hydrogels	Hair regeneration	298
No cell loading	Collagen mimetic peptide (GGYGGGPPC[GPP] ₅ GFOGER[GPP] ₅ GPC) where O is hydroxyproline (3D culture, coating on PCL)	Bone formation	270
Rat BMSCs	Hyaluronic acid hydrogels grafted with adhesive peptide (PPFLMLLKSTR)	SCI injury treatment	299

^aADSCs, adipose-derived stem cells; BMSCs, bone marrow stromal cells; ESCs, embryonic stem cells; hADSCs, human ADSCs; hESCs, human ESCs; gBMSCs, goat BMSCs; hBMSCs, human BMSCs; hiPSCs, human induced pluripotent stem cells; hiPSC-CM, hiPSC-derived cardiomyocyte; hNSCs, human NSCs; mBMSCs, murine BMSCs; NPCs, neural progenitor cells; NSCs, neural stem cells; SCI, spinal cord injury; UC-MSC, umbilical cord-derived mesenchymal stem cells.

chemical crosslinkings, or other stimulation such as pH change, light exposure, or heating. Therefore, this may afford high cytocompatibility in the hydrogels, which avoids potentially harmful UV exposure in photocrosslinking. One of the self-assembly peptides, KLD peptide, is composed of repeating alternative sequences of aspartic acid (D), leucine (L), and lysine (K).³⁰⁰ Single peptide sequences were aggregated under the hydrophobicity of leucine; and nanofibers were extended under the electrostatic force between positively charged lysine and negatively charged aspartic acid, which resembled other amphiphilic self-assembled peptide fibers.³⁰⁰ Nanofibers finally cluster to generate free-standing hydrogels. The self-assembled oligopeptide nanofibers are similar to the fibrous cartilaginous ECMs and give a conducive 3D microenvironment with biomimetic nanoscale architecture for the expansion and chondrogenic induction of hMS cells.^{301–304}

Li and his colleagues designed self-assembled peptide hydrogels that are functionalized with *N*-cadherin mimetic oligopeptide (HAVDI), to investigate the signaling mechanism of enhanced chondrogenesis by the self-assembled oligopeptide.³⁰⁰ The inclusion of the *N*-cadherin oligopeptide in the self-assembled oligopeptide (Ac-HAVDIGGKLDLKLKLDL) hydrogels greatly promoted the expression of chondrogenic markers and cartilaginous matrix generation by encapsulated hBMS cells. This is because *N*-cadherin, a transmembrane protein and major component of adhesion junction, mediates cell–cell interactions and intracellular signaling that are key factors in the control of organ development and cell behaviors. The results suggested that *N*-cadherin mimetic oligopeptide promotes hBMS cell chondrogenesis by restricting the transcription of canonical Wnt signaling from immunofluorescence staining, western blotting, and RT-qPCR experiments, which showed that the conjugated *N*-cadherin oligopeptide increased the expression of GSK-3 β and decreased the nuclear localization of β -catenin.³⁰⁰ This study sheds light on the underlying molecular mechanism of the pro-chondrogenic effect of *N*-cadherin oligopeptide in the context of chondrogenic inductive condition.

Dissanayaka and his colleagues used the self-assembling peptide PuraMatrix™ as a scaffold system to study the role of dental pulp stem (DPS) cells in triggering angiogenesis and the potential for regenerating vascularized pulp *in vivo*.³⁰⁵ DPSCs, HUVECs (human umbilical vein endothelial cells), or co-cultures of both cell types were entrapped in 3D PuraMatrix™ nanofiber hydrogels, which supported cell migration and survival, and capillary network formation in the absence of growth factor addition. DPS cells enhanced early vascular network formation by promoting the migration of HUVECs and by increasing the expression of VEGF (vascular endothelial growth factor).³⁰⁵ Both the DPS cell monoculture and co-culture systems showed vascularized pulp-like tissue with patches of osteodentin after transplantation in mice. The co-cultured group expressed more ECMs, mineralization, and vascularization than the DPS cell monoculture group *in vivo*. The DPS cells played an important role in initial angiogenesis, whereas coordinated efforts by the DPS cells and HUVECs were necessary to

gain a balance between mineralization and ECM deposition.³⁰⁵ These results suggest the importance of a microenvironment that supports cell-cell interactions and cell migration, which contribute to successful dental pulp regeneration.

Hogrebe and Gooch prepared self-assembling peptide hydrogels having tunable stiffness and cell-binding site density as well as a fibrous micro-architecture resembling the structure of COL.²⁸⁵ They studied the effect of culture dimensionality on the differentiation of hBMS cells at different values of matrix stiffness (storage modulus $[G'] = 10, 5, 1.25, \text{ and } 0.25 \text{ kPa}$) and a constant RGD (Arg-Gly-Asp) binding site concentration using Ac-GRGDSP-GG-FKFEFKFE-NH₂ peptide.²⁸⁵ The cultivation on top of stiff gels promoted the most efficient osteogenic differentiation in the presence of the same soluble induction factors, whereas the encapsulation of hBMS cells within the same stiff gels resulted in a switch to predominantly terminal chondrogenic differentiation. Adipogenic differentiation dominated at soft hydrogels, and 3D cultivation of hBMS cells induced their adipogenic differentiation better than 2D culture at a given stiffness.²⁸⁵ The results suggest that the optimal culture conditions corresponding to each cell type highlights the importance of incorporating native matrix dimensionality and stiffness.

2.10.2 Osteogenic Induction on ECM-peptide Immobilized Dishes and Scaffolds

Hennessy and his colleagues examined the interaction between hBMS cells and HYA disks coated with the COL-mimetic oligopeptides GFOGER, GTPGPQGIAGQRGVV (P15), and DGEA.²⁴ hBMS cells attached equally well to disks coated with COL type I, P15, or DGEA, and all three substrates, but not GFOGER, showed higher cell attachment than uncoated HYA disks.²⁴ On the other hand, another study reported that PCL scaffolds coated with GFOGER facilitated bone generation in critically sized segmental defects in rats.²⁷⁰ The combination of appropriate scaffold materials and ECM-oligopeptides would be effective for regulating MS cell induction.

When oligopeptide-coated HYA disks were overlaid with proteins from the tibial microenvironment or serum, COL-mimetic coated HYA disks did not prohibit hBMS cell attachment, while RGD peptide-coated HYA disks did.²⁴ On the other hand, they did not enhance adhesion either. ALP activity and osteocalcin secretion from hBMS cells attached to P15-coated DGEA-coated disks were facilitated by stimulation of COL-selective integrins that activated osteogenic induction.²⁴ Both of these osteogenic markers were elevated by P15 and DGEA in the absence or presence of induction medium. Bone generation on HYA tibial transplants was increased by the COL-mimetic oligopeptides. Then, COL-mimetic oligopeptides enhanced osteointegration of HYA disks, especially by activating osteoblastic induction, rather than attachment of MS cells.²⁴

Poly(ethylene glycol) diacrylate hydrogel-incorporated RGD oligopeptides were found to facilitate osteogenic induction of goat BMS cells,²⁶⁰ although RGD oligopeptide-coated HYA scaffolds did not facilitate osteogenic induction.²⁴ RGD oligopeptides supported BMS cells to retain cbfa-1 expression in the hydrogels. Soluble RGD was reported to significantly inhibit the mineralization of BMS cells, as indicated by von Kossa staining, phosphorus elemental assay, and quantitative calcium assay.²⁶⁰ This study indicated that RGD-conjugated hydrogel facilitated the osteogenic differentiation of BMS cells in a dosage-dependent way, with 2.5 mM being the adequate concentration in their hydrogel preparation.²⁶⁰ The combination of scaffold biomaterials and ECM-oligopeptides seems to effect MS cell induction in the hydrogels and scaffolds.

hBMS cells and porous biodegradable silk scaffolds were used to engineer bone-like tissue *in vitro*.²⁶⁶ Two different scaffolds with the same microstructure were investigated: silk scaffolds with covalently conjugated RGD sequences (to evaluate the effects of increased cell attachment and slow degradation) and COL scaffolds (to evaluate the effects of fast degradation).²⁶⁶ hMS cells were isolated, proliferated in cultivation, and examined with respect to the expression of surface markers and the ability for osteogenic and chondrogenic induction. Cells were inoculated on the scaffold and cultivated for up to 1 month. Microcomputer tomography and histological assay suggested the development of organized, interconnected, and 1.2 mm long bone-like trabeculae with cuboid cells on the silk-RGD scaffolds, which were absent on the COL scaffolds and present to a lesser extent on silk scaffolds.²⁶⁶ The X-ray diffraction pattern of the deposited bone indicated HYA in the natural bone. Biochemical assay indicated the enhanced mineralization on silk-RGD scaffolds in comparison to either COL or silk scaffolds after 1 month.²⁶⁶ Expression of bone morphogenetic protein 2, osteopontin, and bone sialoprotein was much higher in hBMS cells cultivated in osteogenic media than control media after 2 and 4 weeks in cultivation.²⁶⁶ These findings indicate that RGD-silk scaffolds are extremely appropriate for autologous bone regeneration due to their tunable mechanical characteristics and stable macroporous structure, which match those of native bone and slow degradation.²⁶⁶

2.10.3 Chondrogenic Induction on ECM-peptide Immobilized Dishes and Scaffolds

Poly(ethylene oxide) diacrylate (PEODA) hydrogels provide 3D structural support for *in vivo* and *in vitro* chondrogenic induction of stem cells. However, PEODA hydrogels are bio-inert, as are most synthetic scaffolds, and exhibit non-attachment to proteins as well as stem cells.^{306,307} Some investigators have designed PEODA scaffolds conjugated with ECM-oligopeptides, such as RGD peptide^{262–264} and COL-mimetic peptides (CMPs)²⁶¹ or chondroitin sulfate²⁶⁵ for chondrogenic induction of BMS cells.

The COL-mimetic oligopeptides (CMPs) are sequences of $-(\text{Pro-Hyp-Gly})_7-$, where Hyp is hydroxyproline, and they hold a COL-like triple helical configuration, which has associated with COL fibers *via* a strand invasion process.^{308,309} Lee and his colleagues found that the CMP-mediated micro-environment promoted the chondrogenic induction of goat BMS cells. BMS cells were photo-entrapped in the CMP-binding PEODA hydrogel.²⁶¹ Bio-chemical and histological assay of the CMP-binding PEODA hydrogel showed twice as much COL and glycosaminoglycan content as in the control PEODA hydrogel after 21 days.²⁶¹ BMS cells cultivated in CMP-binding PEODA hydrogels showed a lower level of the hypertrophic markers COL type X and cbfa-1 than BMS cells in PEODA hydrogel by examination by immunohistochemistry and gene expression.²⁶¹ These findings show that the CMP-binding PEODA hydrogel provides an adequate microenvironment for entrapped BMS cells and controls their chondrogenic induction.²⁶¹

Hwang and his colleagues studied the chondrogenic ability of hES cell-derived MS cells in pellet cultivation and after entrapment in PEODA hydrogel with exogenous extracellular biomolecules (COL type I and HA) or grafted with RGD oligopeptides.²⁶³ The hES cell-derived MS cells showed growth factor-dependent matrix formation in pellet cultivation but did not generate tissues with characteristic cartilage morphologies. No significant matrix production or cell growth was detected in the PEODA hydrogel including exogenous COL type I or HA.²⁶³ On the other hand, ECM production, cartilage-specific gene up-regulation, and neocartilage with basophilic ECM deposition was found within 21 days of cultivation for hES cell-derived MS cells entrapped in PEODA hydrogel grafted with RGD oligopeptide.²⁶³ These results indicate that precursor cells of a MS cell population from inducing hES cells through EBs can make cartilage tissues using hydrogels grafted with RGD oligopeptide.²⁶³

Betre and his colleagues investigated the ability of a genetically engineered elastin-like polypeptide (ELP) to facilitate chondrocytic induction of hADS cells with no exogenous chondrogenic supplements.²⁶⁸ ELPs have a repeated oligomeric pentapeptide sequence consisting of Val-Pro-Gly-Xaa-Gly (valine-proline-glycine-Xaa-glycine), where Xaa is the guest residue and can be selected from any amino acids with the exception of proline.³¹⁰ ELPs generate clusters in aqueous solution at an appropriate transition temperature, *i.e.*, an inverse temperature phase transition (T_i). Below T_i , ELPs are highly solvated and structurally disordered and, subsequently, soluble in aqueous solution. ELPs undergo desolvation and generate a gelatinous cluster termed a coacervate when the temperature is above T_i .^{268,311} Entrapment of hADS cells in ELP hydrogels can be simply made by ELP coacervate generation.

hADS cells were cultivated in ELP hydrogels in either standard or chondrogenic medium at 5% oxygen for up to 14 days.²⁶⁸ The ELP hydrogels containing hADS cells cultivated in either medium showed greatly enhanced COL and sulfated glycosaminoglycan production, whereas the matrices secreted by hADS cells were composed mainly of COL type II but not COL type I.²⁶⁸ The composition of the ELP hydrogel including hADS cells cultivated in either medium did not differ greatly.²⁶⁸

The ELP hydrogel including hADS cells was cultivated in standard media at either 20% or 5% O₂ for 1 week to examine the effect of oxygen tension on the differentiation of hADS cells in ELP hydrogel. These hADS cells exhibited enhanced COL type II and Sox9 gene expression at both oxygen concentrations, and the gene expression of COL type I was reduced.²⁶⁸ On the other hand, the ELP hydrogel including hADS cells cultivated in 20% O₂ had highly elevated gene expression of COL type X, suggesting hypertrophic conditions, which was not observed in the 5% O₂ cultivation.²⁶⁸ This study indicates that ELP hydrogel can facilitate chondrogenesis of hADS cells in the absence of exogenous dexamethasone and TGF- β 1, especially under low oxygen tension.

A hydrophobic polyhydroxyalkanoate (PHA) scaffold was prepared from a copolymer of PHBHHx, poly(3-hydroxybutyrate-co-hydroxyhexanoate). Some amphiphilic proteins can be immobilized on the interface of PHA granules *in vivo*, such as PHA granule-associated protein (PhaP) and PHA synthase (PhaC).³¹² You and his colleagues generated RGD-PhaP fusion proteins using recombinant gene technology.²⁶⁷ hBMS cells on the PHA scaffold immobilized with RGD-PhaP fusion protein were cultivated to examine the generation of articular cartilage obtained from chondrogenic induction.²⁶⁷ The scaffold immobilized with RGD-PhaP fusion protein induced better cell adhesion, more homogeneous spreading of cells, and better chondrogenic induction in comparison to the scaffolds immobilized with PhaP or uncoated scaffolds in serum-containing media.²⁶⁷ Furthermore, more ECMs were generated by the differentiated cells over 2 weeks on the scaffold immobilized with RGD-PhaP fusion protein that was evaluated by increased expression of chondrocyte-specific genes including COL type II, aggrecan, and SOX9. This finding suggested a positive effect of RGD on ECM production.²⁶⁷ In addition, the sGAG (sulfated glycosaminoglycans) and total COL content, which are cartilage-specific, were generated much more on the scaffolds immobilized with RGD-PhaP fusion proteins than on uncoated scaffolds or the scaffold immobilized with PhaP protein.²⁶⁷ The cartilage-like matrices where chondrocyte-like cells located homogeneously were found on the scaffold immobilized with RGD-PhaP fusion protein after 21 days.²⁶⁷ These findings support the generation of engineered cartilage tissues.

It is difficult to create a hierarchical tissue structure that mimics the extremely organized zonal architecture of articular cartilage, which is composed of four distinct zones spatially: the calcified, deep, transitional (middle), and superficial zones.²⁶⁵ Each zone has unique cellular organization, mechanical properties, and ECM compositions. The cartilage ECMs mainly consist of glycosaminoglycans (GAGs) and COL type II, whose relative concentrations are different spatially from the superficial to the deep zone, leading to varying mechanical properties.^{265,313} The superficial zone contains low levels of GAG and high levels of COL type I.^{265,314} The transitional zone has a higher GAG concentration and a lower COL type II content.²⁶⁵ The deep zone contains the lowest level of COL type II fibers and the highest concentration of GAGs.^{265,315} The calcified cartilage zone integrates the cartilage to the subchondral bone and contains high levels of COL type X.^{265,314,315}

Nguyen and his colleagues reported that different combinations of natural and synthetic biopolymers generated unique environments that could guide BMS cells to induce into the transitional, superficial, and deep zones of articular cartilage.²⁶⁵ PEG hydrogel blended with MMP-pep (matrix metalloproteinase-sensitive peptides) and CS (chondroitin sulfate), CS:PEG:MMP-pep, induced low levels of proteoglycan and high levels of COL type II expression, which resulted in a low compressive modulus, which was similar to the superficial zone.²⁶⁵ PEG hydrogel blended with CS (CS:PEG) generated intermediate levels of both proteoglycans and COL type II as in the transitional zone, while PEG hydrogel blended with HA, HA:PEG, generated low COL type II and high proteoglycan levels with a high compressive modulus, similar to the deep zone.²⁶⁵ The compressive moduli of these zone-specific matrices following cartilage formation indicated a similar trend to the corresponding zones of articular cartilage, with CS:PEG:MMP-pep having the lowest compressive modulus, then CS:PEG, and HA:PEG having the highest modulus.²⁶⁵ These findings demonstrate the ability of composite scaffold structures with BMS cells and material compositions to form zonally organized and functional articular cartilage-like tissues.

2.10.4 Neural Induction on ECM-peptide Immobilized Dishes and Scaffolds

Cellular adhesive motifs can be designed into the extracellular loops of outer membrane protein A (OmpA). Cooke and his colleagues designed outer membrane proteins to generate SAMs (self-assembled monolayers) on a gold interface where the proteins were adequately oriented on a gold interface, which enable the presentation of the oligopeptide in a highly regulated manner.²² The cellular adhesion motifs used in their research were: YIGSR from LN, MNYYSNS from COL type IV, P15 (GTPGPQGIAGQRGVV) from COL type I, and PHSRN and RGDS from FN.^{22,109,121,259} Adult NSP cells cultivated on monolayers of OmpA inscribed with FN (PHSRN) and COL type I (P15, GTPGPQGIAGQRGVV) motifs induced into beta-III tubulin positive cells, while the cells on OmpA inscribed with COL type IV could not.²² This finding indicates how a biomimetic protein interface expressing the active peptide domains of ECM proteins can control the neural induction of stem cells *in vitro*.

Varun and his colleagues designed a vitronectin-derived peptide (VDP, CGKKQRFRRNRKG), which served as an adhesive coating material for the long-term expansion of several human NPC lines.²⁷⁵ VDP-coating dishes allowed for the directed neuronal differentiation of human NPCs at levels similar to the cells differentiated on a traditional LN-coating interface.²⁷⁵ Overall, the ability of VDP to support the long-term expansion and directed neuronal differentiation of human NPCs may significantly advance future translational medicine using human NPCs in treatment of disorders, injuries, and diseases of the CNS.

N-cadherin is a cell–cell adhesive molecule and plays key functions in neural generation. Yue and his colleagues designed an artificial ECM to mimic *N*-cadherin-mediated cell attachment.³¹⁶ They prepared chimeric proteins, which included the Fc domain of immunoglobulin G (IgG) and extracellular domain of *N*-cadherin, *N*-cad-Fc protein.³¹⁶ *N*-cad-Fc proteins could extensively attach to the hydrophobic interface. Both MEB5 (neural stem) and P19 (embryonal carcinoma) cells cultivated on *N*-cad-Fc protein-coated interfaces displayed scattering morphologies with no colony generation and greater proliferation than typical cultivation systems, by keeping their pluripotent state.³¹⁶ Both cell lines cultivated on an *N*-cad-Fc protein-coated interface also induced into neural cells with the single cell morphology when differentiated with appropriate conditions.³¹⁶ It was found that the *N*-cad-Fc protein would be used as an artificial ECM protein for stem cell cultivation.³¹⁶ E-cad-Fc protein (recombinant E-cadherin fusion protein having an IgG Fc region) was also made by Haque and his colleagues.²⁵⁶ ES cell cultivation on plates immobilized with E-cad-Fc protein could efficiently induce into hepatic cells with morphologies of single cells. The recombinant ECMs might be efficiently used as an *in vitro* model for investigating the mechanisms of early phases of liver development of ES cells at the single cell level.²⁵⁶

2.11 Biomaterials with *N*-Cadherin Mimicking Oligopeptides

The cell–cell signaling pathway *via* *N*-cadherin plays a key role in the structure and function of the nervous system. Several studies report that *N*-cadherin promotes neurite growth, which is similar to LN.³¹⁷ Neuronal differentiation of stem/progenitor cells on the *N*-cadherin surface reduced the number of astrocytes in culture,³¹⁸ although some studies reported that *N*-cadherin is not sufficient for cell adhesion^{319,320} and other bioactive molecules promote better neurite extension than *N*-cadherin.³²¹ This indicates that *N*-cadherin has an important function in neural tissue development and needs more investigation to be better understood. Therefore, Lim and his colleagues prepared a continuous gradient of *N*-cadherin oligopeptide, His-Ala-Val-Asp-Lle (HAVDI) in polyethylene glycol dimethacrylate (PEGDA) hydrogels and investigated concentration-dependent effects of *N*-cadherin oligopeptide on survival and neural induction of murine ES cells.³²²

The *N*-cadherin oligopeptide was observed to contribute to the expression of pluripotency marker (ALP activity, Oct3/4, and Nanog) in murine ES cells cultivated on PEGDA hydrogels containing *N*-cadherin peptide in a concentration-dependent manner.³²² *N*-cadherin oligopeptide concentrations in the PEGDA hydrogels expressed a biphasic response in neurite extension length and mRNA expression of neural differentiation marker β III tubulin (TUB1) in murine ES cells cultivated on the PEG hydrogels containing *N*-cadherin oligopeptide (Figure 2.13).³²² High concentration of *N*-cadherin oligopeptide in the PEGDA hydrogels increased the expression of apoptotic

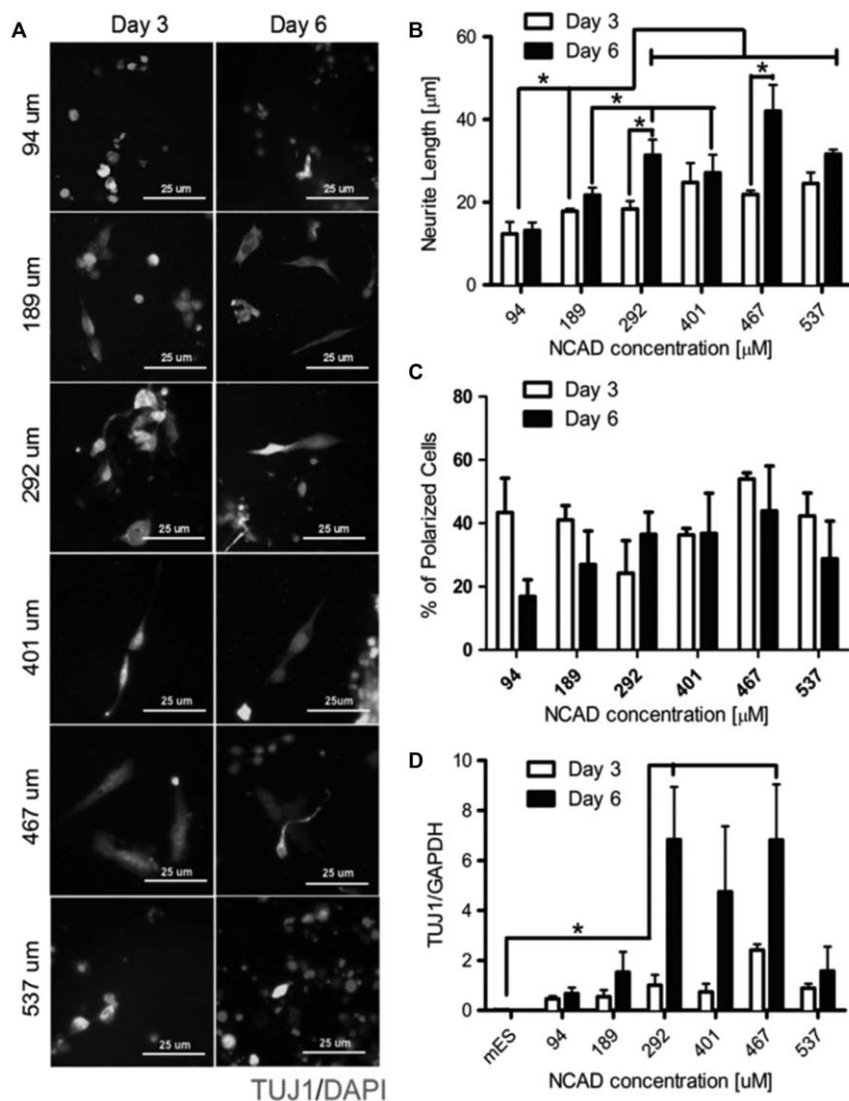


Figure 2.13 Neural induction of murine ES cells cultivated on polyethylene glycol hydrogels including a continuous *N*-cadherin peptide (NCAD) concentration gradient. (A) Immunofluorescent staining of TUJ1 (neuron-specific class III β -tubulin) and nuclei. (B) Quantification of average neurite length, (C) Percentage of polarized cells, (D) TUJ1 gene expression.³²² Adapted from ref. 322 with permission from Elsevier, Copyright 2017.

marker, caspase 3/7, in murine ES cells compared to that of murine ES cells cultured on PEGDA hydrogels containing a lower concentration of *N*-cadherin peptide.³²²

Increasing the *N*-cadherin oligopeptide concentration in the PEGDA hydrogels facilitated higher survival of murine ES cells, which were exposed to increasing oxidative stress originated by exposure to hydrogen peroxide.³²²

It is important to understand the effects of *N*-cadherin signaling, so that it can be widely used in tissue engineering matrix design to promote the restoration of neurological function in those receiving stem cell therapy after traumatic damage to the CNS.

2.12 Conclusion and Future Perspective

ECM proteins not only work as supporting biomaterials for stem cells but also support to control cellular functions, especially determination of stem cell fate.^{238,323} Moreover, ECM proteins can regulate signal transduction generated by various bioactive molecules, including growth factors.^{238,324} The morphologies of MS cells are controlled by regulating the attachment of cells to ECM proteins, and cell morphologies can, in turn, control cell induction. The interaction between MS cells and specific ECM proteins can regulate induction of MS cells into specific lineages.

Decellularized ECM scaffolds are attractive materials, because these scaffolds can retain the design of the original tissues and form biological environments more accurately than scaffolds made from single ECMs. Decellularized ECM scaffolds might be powerful tools for the induction of MS cells into several difficult lineages, such as hepatocytes, dopamine-secreting cells, and beta cells.

Natural or synthetic biopolymers including ECM-oligopeptides are promising materials for scaffolds or hydrogels including MS cells. A variety of biomaterial designs for scaffolds and hydrogels including MS cells are possible, using biopolymers that have ECM-oligopeptides, which allow cell attachment, expansion and induction into specific lineages. On the other hand, it is currently complicated to describe the direction of desired induction lineages from the interaction of MS cells and appropriate ECM-oligopeptides. The combination of base biopolymers and ECM-oligopeptides on scaffolds, as well as the physical and chemical properties of scaffolds, determine the induction of MS cells into desired lineages.

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CHAPTER 3

Feeder-free and Xeno-free Culture of Human Pluripotent Stem Cells on Biomaterials

3.1 Introduction

Human pluripotent stem (hPS) cells, including human induced pluripotent (hiPS) cells^{1,2} and human embryonic stem (hES) cells,^{3–5} are promising for drug discoveries, disease modeling and regenerative medicine. In order to fully utilize hPS cells in tissue engineering and cell therapy, the advancement of a well-defined microenvironment for culturing hPS cells is needed.^{6–13} The present highest quality level for proliferation and maintenance of hPS cells is typically in a state that is no different whether it involves cultivation on feeder cells (*e.g.*, MEFs or human feeder layers prepared with human adipose-derived cell¹⁴ and human foreskin fibroblast^{15,16}) or on Geltrex^{17,18} and Matrigels^{19–23} (Figure 3.1).²⁴ The use of feeder layers (cells) to cultivate hPS cells is a laborious process, which varies depending on the specific lots of feeder cells or skill of preparation of feeder cells. In contrast, Geltrex and Matrigels are made of molecules extracted from mice sarcomas of Engelbreth–Holm–Swarm, which contain enactin, heparan sulfate proteoglycan, collagen (COL) IV, and LN as well as several growth factors such as TGF- β (transforming growth factor- β), FGF (fibroblast growth factor), and EGF (epidermal growth factor). The hPS cell culture on the dishes coated with Geltrex or Matrigels is the typical and most reliable method to keep the pluripotent states of many hiPS and hES cell lines in feeder-free conditions (Figure 3.1).²⁵ However, these culture conditions are not chemically

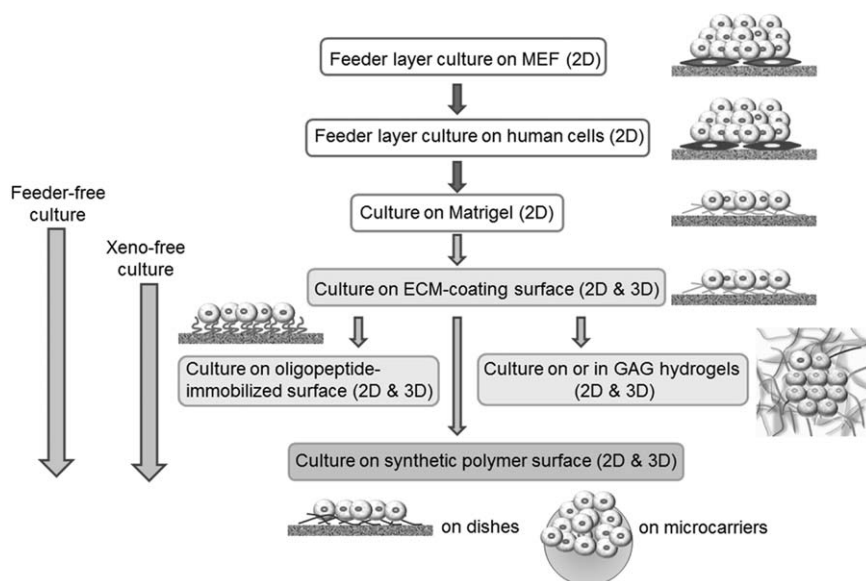


Figure 3.1 Human pluripotent stem (hPS) cell cultivation method. hPS cells can be cultivated on human feeder cells or MEFs (mouse embryonic fibroblasts). Feeder-free cultivation of hPS cells is available on xeno-containing Matrigel-coated dishes. Some types of xeno-free and feeder-free cultivation of hPS cells are studied on ECM-coated materials and peptide-immobilized materials. hPS cells are cultivated on synthetic polymer surfaces or on polysaccharide hydrogels by choosing an appropriate polymer or an appropriate GAG with adequate water content.²⁴

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defined and contain xeno-derived molecules because Geltrex or Matrigels are prepared from the extract from mice. Their xeno-derived molecules hinder the clinical use of hPS cells cultured on Geltrex or Matrigel coated dishes.^{26,27} In particular, there are concerns for immunogenic epitopes to humans (*e.g.*, the non-human sialic acid *N*-glycolylneuraminic acid [Neu5Gc]), as well as the transfection of xenogenic viruses originated from Geltrex or Matrigels.²⁶

It is critical to develop cell culture biomaterials that support large-scale production of hES and hiPS cell lines under xeno-free and feeder-free systems, which are compliant with cGMP (current good manufacturing practice).^{28–35} The use of feeder layers restricts the use of hPS cells in clinics. Many studies have reported several alternative hPS cell cultivation methods that have no feeder layers. In any case, the most important parameters that determine the supporting self-renewal properties of hPS cells are not completely clear.^{20,36–38} The basic FGF (FGF-2), activin/nodal and Wnt pathways are considered to be critical to support the pluripotency of hPS cells. Furthermore, regulated expansion that supports the pluripotency of hPS cells is

a critical factor to create the bankable populations of hPS cells, which are required for clinical application as well as future investigation geared toward regenerative therapies.³⁶

Currently, the combination of optimal cell culture materials (recombinant VN [rVN]-coated or LN 521 [LN521]-coated dishes) and xeno-free and chemically defined cell culture media containing FGF-2, TGF- β , and transferrin (e.g., Essential 8) can be used for hPS cell culture as a gold standard of feeder-free and xeno-free culture. However, long-term cultivation of hPS cells in this condition is still unknown. Therefore, mother cell lines of hPS cells are typically kept to be cultivated on Matrigel-coated dishes and not only on rVN or LN-521-coated dishes for research into the use of hPS cell lines.

Recently, several cell cultivation matrices were reported to cultivate hPS cells that support their pluripotency in chemically defined media. The chemically defined medium indicates that the medium is not composed of calf serum or fetal bovine serum. In the past, knockout serum replacement (KSR) was used in hPS cell culture, but the medium containing KSR is now rarely used. Furthermore, leukocyte inhibitory factor (LIF) was added to the culture medium of hPS cell culture in the past, because mouse ES cells can be cultured on gelatin-coated dishes in the medium containing LIF. However, we had recognized that LIF does not support the pluripotency of hPS cells. Therefore, recent studies dealing with ES or iPS cells using LIF in the cell culture medium are only investigating properties of mouse ES or iPS cells and not of human ES or iPS cells.

Table 3.1 summarizes ECM-immobilized surfaces for hES and hiPS cell culture on ECM-coated surfaces in chemically defined and/or without xenogenic substrates.^{18,28,39–66} Furthermore, Table 3.2 summarizes glycosaminoglycan-immobilized, chimera protein-immobilized, and oligopeptide-immobilized biomaterials for hES and hiPS cell culture on ECM-coated surfaces in chemically defined and/or devoid of xenogenic substrates.^{27,67–91} Currently, the developed biomaterials need a combination of specific cultivation media, and specific hPS cells may specifically support their pluripotent state on the cell cultivation biomaterials.⁴⁷ Moreover, it is now difficult to choose the ideal and best biomaterials for hPS cell cultivation, although rVN and LN-521 are starting to be used as a gold standard of cell culture matrices for hPS cell culture.

This chapter describes in detail the current developments in hPS cell cultivation biomaterials and discusses the material-assisted regulation of hPS cells under xeno-free and feeder-free cell cultivation conditions.

Some strategies can be considered for development of materials for hPS cell cultivation under chemically defined, xeno-free and feeder-free systems. The strategies are (1) hPS cell cultivation on two-dimensional (2D) biomaterials immobilized natural extracellular matrices (ECMs), (2) on or within 2D or 3D hydrogels made from polysaccharide such as GAG (glycosaminoglycan), (3) on 2D biomaterials immobilized synthetic oligopeptides derived from ECMs, (4) on 2D plates made from synthetic polymers, (5) in

Table 3.1 Feeder-free cultivation of hPS cells on ECM-containing substrates in defined media.²⁴ Adapted from ref. 24 with permission from Elsevier, Copyright 2014.^a

Cell lines	Cell culture substrates (2D or 3D culture)	Culture medium	Longest time in culture	Ref. (year)
hESCs (H9, CA2)	Decellularized ECMs from human foreskin fibroblasts (2D)	HEScGRO with bFGF	20 passages	39 (2010)
hESCs (H1, H9)	Decellularized ECMs from human EB derived from hESCs (2D)	TeSR2	20 passages	40 (2011)
hESCs	Decellularized ECMs from human foreskin fibroblasts (2D)	TeSR2	Not specified	28 (2012)
hiPSCs (UTA1)	FN-coating dishes (2D)	hESF9a	27 passages	41 (2010)
hESC (16)	CELLstart-coating dishes (2D)	StemPro	28 passages	18 (2009)
hESCs (Shf3, Shf6)	CELLstart-coating dishes (2D)	StemPro	20 passages	43 (2011)
iPSC from ADSCs	CELLstart-coating dishes (2D)	NutriStem	Not specified	44 (2010)
hESCs (RC6, 9, 10, 13)	CELLstart-coating dishes (2D)	StemPro	Not specified	45 (2012)
hESCs (H1, HSF1)	FN or LN-coating dishes (2D)	DMEM/F12 with small molecules and bFGF	25 passages	46 (2011)
hESCs (H1, H9, CA1) iPSC (4YA, 4YE, BJ-EOS, 4YF)	FN, VN, Col I, PDL, or CELLstart-coating dishes (2D)	HGM, XSR, or TeSR2 with and without Y27632	15–34 passages	47 (2012)
hESCs (H9, CHA6)	CELLstart or Vitronectin-coating dishes (2D)	mTeSR1 or StemPro	6 passages	48 (2010)
hESCs (H9, CA1)	CELLstart, StemXVivo, BridgeECM, MEF-CMTX, or FN-coated dishes (2D)	mTeSR1	5–10 passages	49 (2011)
hESCs (HES2, HUES1)	VN, LN, FN, Col IV, or entactin-coating dishes (2D)	mTeSR1	8 passages	50 (2008)
hESCs (HS181, HS237, HS293, HS306)	Mixed ECMs (human Col IV, VN, FN, LN)-coating dishes (2D)	mTeSR1, X-Vivo 20	Not specified	51 (2007)
hESC (BGO1V)	LN-coating dishes (2D)	DMEM/F12, Activin-A, bFGF, chimera protein	35 passages	52 (2010)
hESCs (HS207, HS420, HS401)	LN-511 coating dishes	O3 or H3 medium	20 passages	53 (2010)
hESCs (HES-3, H7)	LN or VN coating microcarriers (3D)	StemPro	20 passages	64 (2012)
hESCs (HES-3, H1)	VN-coating dishes	mTeSR1	20 passages	62 (2011)
hESCs (MEL1, MEL2, hES3)	Recombinant VN	StemPro	10 passages	61 (2010)

^aCol, collagen; EB, embryonic body; FN, fibronectin; LN, laminin; PDL, poly-D-lysine; VN, vitronectin.

Table 3.2 Feeder-free cultivation of hPS cells on peptide-containing, chimera protein-containing, glycosaminoglycan-containing, and synthetic polymer biomaterials in defined media.²⁴ Adapted from ref. 24 with permission from Elsevier, Copyright 2014.^a

Cell lines	Cell culture substrates (2D or 3D culture)	Culture medium	Longest time in culture	Ref. (year)
Synthetic oligopeptide- and polypeptide-immobilized surface				
hESCs (H1, H7, H9, H149), iPSCs (IMR90-1)	Oligopeptide (heparin-binding domain) immobilized dishes (2D)	mTeSR1 medium with ROCK inhibitor	17 passages	67 (2010)
hESCs (H1, H7)	Oligopeptide (BSP, VN)-immobilized dishes (2D)	XVIVO10 + GFs, mTeSR1	10 passages	68 (2010)
hESCs (H9, H14)	Cyclic RGD-immobilized dishes (2D)	mTeSR1	5 days	78 (2010)
hESCs (H9, BGN1)	PDL-immobilized dishes (2D)	mTeSR1 medium with ROCK inhibitor	2 passages	81 (2008)
hESCs (H1, H9)	PDL-coating pDTEc microfibers (3D)	mTeSR1	14 days	82 (2012)
iPSCs	Pronectin F	ReproFF	45 passages	83 (2012)
Chimera protein-immobilized surface				
hESC (H1, H9), hiPSC (hiPSC3a, hiPSC6a)	E-cadherin chimera	mTeSR1	35–53 passages	84 (2010)
hESCs	StemAdhere (chimera E-cadherin)-coating dishes (2D)	TeSR2	Not specified	85 (2012)
GAG scaffold				
hESCs (HUES7, BG01V/hOG), hiPSCs (PD-iPS5, hFib2-iPS4)	Microfiber of chitin/alginate	mTeSR1	10 passages	86 (2012)
Synthetic polymer surface				
hESCs (H9, BG01)	PMEDSAH surface	StemPro	3–10 passages	87 (2010)
hESCs (HUES1, HUES9)	PMVE- <i>alt</i> -MA surface	StemPro	5 passages	88 (2010)
hESCs (H7, H9, BG01, CHB-8, CHB-10)	PMEDSAH surface	StemPro	10 passages	27 (2011)
hESCs (H1, H9)	APMAAm surface	mTeSR1	20 passages	89 (2011)
hESCs (BG01, H9)	PMEDSAH surface	mTeSR1, StemPro	25 passages	90 (2012)
hESC (RH1)	AEtMA-Cl/DEAEA	mTeSR1	20 passages	91 (2013)

^aAPMAAm, aminopropylmethacrylamide; BSP, bone sialoprotein; GAG, glycosaminoglycan; PMEDSAH, poly[2-(methacryloyloxy)ethyl] dimethyl-(3-sulfo)propyl)ammonium hydroxide; PMVE-*alt*-MA, poly(methyl vinyl ether-*alt*-maleic anhydride); VN, vitronectin.

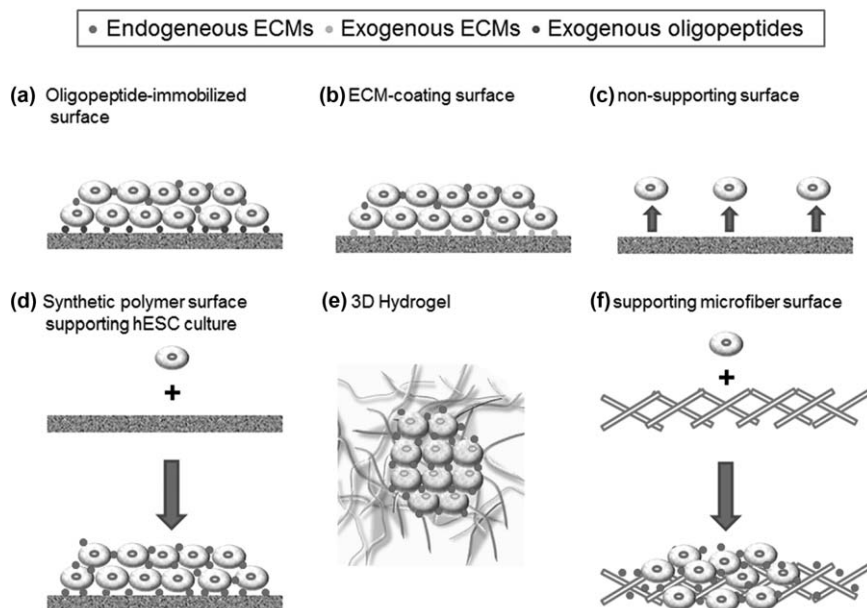


Figure 3.2 hPS cell cultivation model. hPS cell cultivation on (a) peptide-immobilized materials (3D and 2D), (b) ECM-immobilized materials (3D and 2D), (c) materials that do not support hPS cell cultivation (3D and 2D), (d) synthetic polymeric materials that support hPS cell cultivation (3D and 2D), (e) 3D hydrogels composed of GAG or another polymer, and (f) microfibers that support hPS cell cultivation. On the peptide-immobilized (a) and ECM-coated materials (b), which include exogenous peptides and ECM, respectively, hES cells attach through oligopeptide-cell or ECM-cell contacts. hES cells further generate endogenous ECM to make extensive matrix-cell contacts and colonies. On synthetic (d, f) and hydrogel (e) materials that do not have exogenous ECM, hES cells generate ECMs (endogenous ECMs) and generate proximal cell-cell contacts instead of forming extensive matrix-cell contacts and colonies.²⁴

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porous or hydrophilic 3D microcapsules, and (6) on 3D microfibers with or without ECM immobilization (Figures 3.1 and 3.2).

3.2 Analysis of the Pluripotency of hPS Cells

hPS cells show several surface markers of pluripotency, such as Tra-1-81 (tumor rejection antigen 1-81), Tra-1-60, Nanog, Sox2, Oct-4, Oct3/4, SSEA-4 (glycolipid stage-specific embryonic antigen 4), and SSEA-3, but no expression of SSEA-1 (Table 3.3).²⁵ Undifferentiated mouse ESCs show SSEA-1, whereas SSEA-1 is a differentiated marker of hPS cells. hES cells also express large amounts of pluripotent genes, *e.g.*, hTERT, Rex-1, Sox-2, Oct-4, Oct3/4,

Table 3.3 Evaluation of pluripotent hPS cells by protein and gene expression analysis.²⁵ Adapted from ref. 25 with permission from the American Chemical Society, Copyright 2011.

Characterization	Specification (example)
Surface marker analysis of hPSCs	Oct-3/4, Oct-4, Nanog, TRA-1-60, Tra-1-81, SSEA-3, and SSEA-4
Immunohistochemical analysis of hPSCs	Oct3/4, Oct-4, Sox-2, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, and Nanog SSEA-1 (negative staining)
Pluripotent gene analysis of hPSCs	Oct3/4, Oct-4, Sox-2, Nanog , TDGF-1, UTF-1, REX1, hTERT, ABCG2, DPPA5, CRIPTO, FOXD3, Tert1, Rex2, and DPPA5
Differentiation protein analysis in EB and teratoma	(a) Endoderm protein expression AFP, PDX-1, HNF3 β , CK19, glucagon, NFH, GFAP, IFABP, albumin, Titf1, TTF-1, CXCR4, and FOXa2 (b) Mesoderm protein expression cTcN, α -SMA, brachyury T, vimentin, actin, α -actinin, BMP-4, and cTnI (c) Ectoderm protein expression NCAM, nestin, Tuj1, neurofilament, β III-tubulin, GFAP, and enolase
Differentiation gene analysis in EB and teratoma	(a) Endoderm gene expression AFP, albumin, cerberus, GATA3, GATA4, GATA6, SOX17, ONECUT1, FOXA1, FOXA2, IPF1, PROX1, HHX, HNF3b, HNF4a, PDX1, amylase, TTF-1, IFABP, and Titf1 (b) Mesoderm gene expression brachyury T, Hand1, IGF2, FLK1, MIXL1, MESP1, EOMES, PAX3, MYOD1, PECAM1, NKX2, GATA1, GATA2, GATA4, KDR, BMP4, SIL, HOXB4, MyoD, Msx1, C-actin, β -globin, cardiac actin, VE-cadherin, enolase, MtoD, and CD31 (c) Ectoderm gene expression SOX-1, PAX6, nestin, Tuj1, MAP2, NeuroG1, β III-tubulin, NeuroD, NOG, NEFL, keratin, NFH, and neurofilament (NF)-68

and Nanog (Table 3.3).²⁵ Figure 3.3 shows typical analytical processes to characterize the pluripotency of hPS.

The pluripotency of hPS cells is typically characterized from (1) the colony formation observed under light microscopy, whether hPS cells exist as colonies with tight boundaries; (2) the activity of ALP (alkaline phosphatase) evaluated by colorimetric measurements from enzymatic reaction; (3) pluripotency gene expression measured by qRT-PCR and/or RT-PCR measurements; (4) pluripotent protein expression characterized by immunostaining and flow cytometry assay; and (5) the differentiation potential of the cells into three germ layer-derived cells characterized from the formation of embryoid bodies (EBs) (*in vitro* assay) and by teratoma generation (*in vivo*

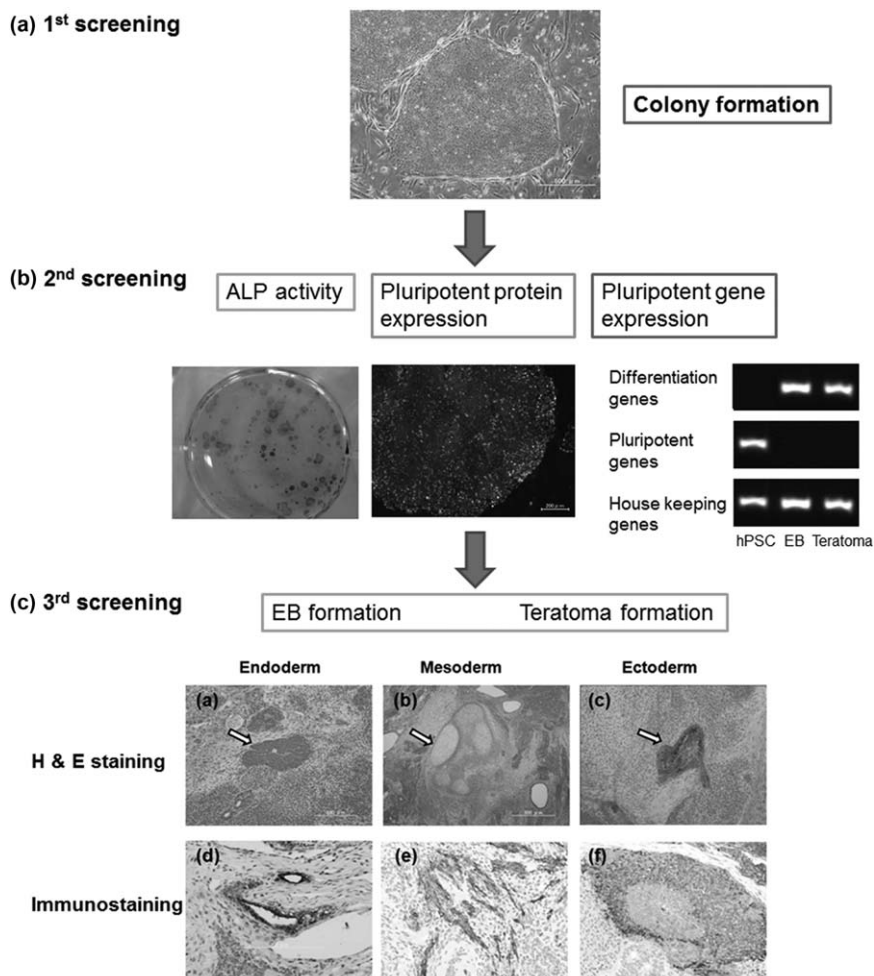


Figure 3.3 Evaluation of pluripotent hPS cells. The pluripotent state of hPS cells is characterized from (a) the colony morphologies observed from microscopy (first screening); (b) the pluripotent gene expression evaluated using RT-PCR or qRT-PCR analysis, the pluripotent protein expression evaluated using flow cytometry analysis and/or immunohistochemical analysis, and the ALP activity evaluated using an enzyme reaction (second screening); and (c) the potential to induce differentiation into cells from all three germ layers evaluated using teratoma and/or EB formation.²⁵

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assay), which are evaluated from H&E (hematoxylin and eosin) staining and immunohistochemical staining assays (Figure 3.3). It is valuable to evaluate whether the hPS cells possess any abnormality of karyotype after long-term cultivation of hPS cells.

3.3 2D Cultivation of hPS Cells on Biomaterials

3.3.1 hPS Cell Cultivation on ECM-immobilized Surfaces in 2D

Although some human cell lines were found to be adequate feeders for hPS cell cultivation to avoid the use of MEFs for xeno-free culture conditions, researchers have started to develop biomaterials with feeder-free conditions using several specific ECMs for hPS cell culture to avoid the use of feeder cells in xeno-free culture conditions. The adhesion-based cultivation of hPS cells relies on the specific ECMs secreted by the feeders for proliferation and attachment. From this consideration, many investigators started to use the ECM molecules produced by feeder cells. Two kinds of ECM are considered for use of hPS cell cultivation: (1) decellularized ECMs secreted by human dermal, fibroblasts from foreskin, adipose-derived stem cells, and other human cell lines that are xeno-free and not chemically defined conditions^{28,36,39,40,92} and (2) mixed or single materials of chemically defined ECM, *e.g.*, natural or recombinant vitronectin (VN), laminin (LN) and fibronectin (FN). The ECMs are used as a coating for biomaterials on cell cultivation plates or scaffolds or dishes. Matrigels are known to maintain the proliferation of hPS cells and support their pluripotent state with no utilization of feeders. Matrigels, which are extracted from mouse Engelbreth-Holm-Swarm (EHS) sarcoma cells, are composed of heparan sulfate proteoglycans, COL IV and LN, *etc.*^{25,51,52,93}

Fu and colleagues developed decellularized ECMs from feeder cells that were derived from EBs, which were differentiated from hES cells *via* a freeze-thaw process (Table 3.1).⁴⁰ ECMs made up of FN, LN, and COL IV would be used to maintain the long-term proliferation of hES cells with xeno-free TeSR2 media for 20 passages.⁴⁰ Therefore, this research indicates another thought for the proficient proliferation of clinically compliant hES cells in feeder-free and autologous cultivation systems.

Typically, hPS cell lines were obtained *via* cultivation on MEFs during their initial development. In other words, these hPS cell lines make it difficult to apply for the clinical therapy of human patients. Ilic and colleagues developed hES cells on decellularized ECMs derived from human neonatal foreskin fibroblasts under xeno-free and feeder-free systems (Table 3.1).²⁸ Their particular transformation (myotonic dystrophy 1 and Huntington's disease)-carrying hES cell lines kept pluripotency on the decellularized ECMs, whereas cell culture plates immobilized with commercially accessible CELLstart (mixture of serum albumin and FN) could not maintain the proliferation of these hES cells in serum-free StemPro media.⁹⁴ On the other hand, conventional hES cell lines can support pluripotency on CELLstart-coated dishes in the StemPro media in general.²⁸ Therefore, it is important to select an appropriate combination of cell cultivation matrices and media, which depends on the specific properties of particular hPS cells.⁴⁷ It appears that no satisfactory coating

Table 3.4 ECMs immobilized on dishes for proliferation, differentiation, and adhesion of stem cells and binding sites of cells.⁹⁵ Adapted from ref. 95 with permission from American Chemical Society, Copyright 2012.

ECM	Binding site of cells
Collagen I	Integrin (α V β 3, α 2 β 1)
Collagen I	Integrin (α 1 β 1)
Collagen I	Integrin (α 1 β 1, α 2 β 1, α 3 β 1)
Collagen II	Integrin (α 1 β 1, α 2 β 1, α 10 β 1)
Collagen IV	Integrin (α 2 β 1, CD44)
Fibronectin	Integrin (α 4 β 1, α 5 β 1, α V β 3, α IIb β 3, α V β 6, α V β 5)
Laminin	Integrin (α 1 β 1, α 2 β 1, α 3 β 1, α 6 β 1, α 6 β 4)
Laminin-1 (laminin 111)	Integrin (α 1 β 1, α 2 β 1, α 6 β 1, α 7 β 1, α 9 β 1), α -dystroglycan, sulfide, and heparan sulfate proteoglycan
Laminin-5 (laminin 332)	Integrin (α 2 β 1, α 3 β 1, α 6 β 1, α 6 β 4)
Laminin-511	Integrin (α 6 β 1)
Laminin-10/11	Integrin (α 3 β 1, α 6 β 1, α 6 β 4)
Vitronectin	Integrin (α V β 3, α V β 5)

biomaterials or cell culture plates exist that can maintain the pluripotent state of hPS for a long time (*i.e.*, >20–30 passages).

Recombinant LN (LN-521 and LN-511) and VN have been used instead of decellularized ECMs or Matrigels for the feeder-free culture of hPS cells. This is because their chemical properties are defined (Table 3.1). Table 3.4 summarizes the ECMs that are immobilized on cell culture plates for cells to provide binding sites.⁹⁵

3.3.1.1 FN (*Fibronectin*)

FN is a glycoprotein of molecular weight around 440 kDa, which binds to other ECMs such as heparan sulfate proteoglycans, fibrin, and COL. FN is composed of a dimer protein that consists of two almost identical monomers connected by a pair of disulfide bonds.

Some researchers found that FN-coated surfaces retain the pluripotency of hES cells (Table 3.1),^{43–46,48,49,96,97} whereas others reported unfavorable data on the cultivation of hES cells on FN-coated surfaces.^{47,50} Amit and colleagues cultivated several hES cell lines (H-9, I-6, and I-3) on FN-coated plates (5.0 $\mu\text{g cm}^{-2}$) in KO-DMEM (knockout DMEM) containing 15% SR (serum replacement). The receptor for integrin, α 5 β 1, which is FN-specific, was expressed in the pluripotent hES cells (Figure 3.4). The hES cells supported pluripotency for more than 6 months and moreover, the hES cells cultivated on gelatin-immobilized plates appeared to be in differentiation.⁹⁶ Human FN was a more favorable ECM for supporting the pluripotent states of the hES cells compared to bovine FN.⁹⁶ SR includes Albumax, which is a lipid-enriched bovine serum. Accordingly, this study was not done under xeno-free cultivation systems.

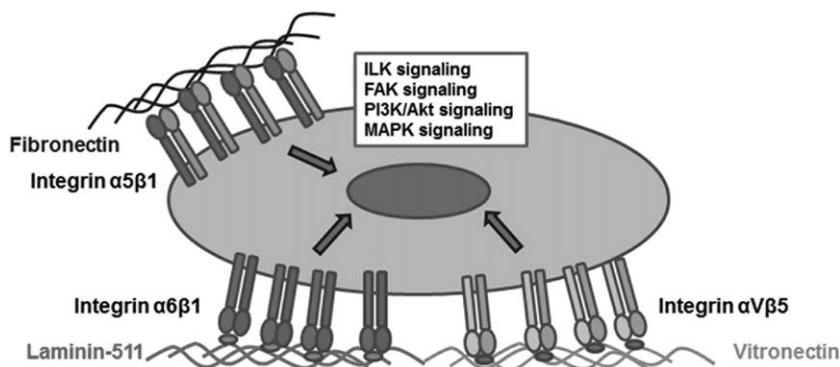


Figure 3.4 hES cell attachment on an ECM-immobilized surface. The attachment of hES cells to LN-511, VN, and FN is mediated by $\alpha_6\beta_1$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$, respectively.²⁴
Adapted from ref. 24 with permission from Elsevier, copyright 2014.

Hughes and colleagues studied financially accessible cell cultivation matrices for hPS cells, such as Bridge Human ECMs (Global Stem), StemX-Vivo (R&D Systems), and CELLstart (Invitrogen), in addition to Matrigels and human basement extract (Table 3.1).⁴⁹ Bridge Human ECMs are composed of FN isoform-1 and albumin, which is similar to the components of CELLstart. StemXVivo is made of a blend of recombinant proteins and is fundamentally made up of VN and enactin.⁴⁹ Hughes and colleagues found that hES (CA1 and H9) cells could retain their pluripotency on FN-coated plates and in addition on plates coated with commercially accessible composite ECMs in mTeSR1 media together with IGF, transferrin, FGF-2, TGF- β_1 , and BSA (bovine serum albumin). Currently, any plates coated with these ECMs cannot maintain and promote the growth of H9 cells with the same stability and doubling time as dishes coated with Matrigels in mTeSR1 media.⁴⁹ The authors commented that FN, either with or without other defined ECMs, had the ability to support the undifferentiated state and pluripotency of hES cells in mTeSR1 medium to some extent.⁴⁹ But, FN on its own cannot stand for optimal ECMs for the cultivation of undifferentiated hES cells based on the results. Several investigators have used CELLstart as a coating substrate for hPS cell adhesion *in vitro*.^{43,44,97,98} Hernandez and colleagues cultivated hES (Shef6 and Shef3) cells on CELLstart-coated dishes in StemPro medium where hES cells kept their pluripotency and expressed normal karyotyping for 20–22 passages (Table 3.1).⁴³ Some investigators also succeeded in the proliferation of hES cells from a single cell to clonal expansion.⁴³ The clonal expansion of hES cells is important for the safety determination of hPS cells for clinical treatment, most importantly if there is a genetic change in the cells.

A feeder-free preparation of hiPS cells from human adipose-derived stem (hADS) cells on CELLstart-coated dishes was investigated by Sugii and

colleagues (Table 3.1).^{44,97} The cells were successively reprogrammed into hiPS cells on the dishes, albeit more time was needed to produce hiPS cell colonies *via* the feeder-free reprogramming method than for the feeder-dependent method.⁴⁴ Hayashi and colleagues reported reprogramming of adult human dermal fibroblasts into hiPS cells under defined and feeder-free cultivation systems (Table 3.1).⁴¹ hiPS cells were cultivated on FN-coated plates in hESF9a-based media. Due to the way xeno-free media and matrices were being used, the established hiPS cell line expressed little or no Neu5Gc, which generates immuno-rejection when the hPS cells are transplanted into human patients.⁴¹ The data indicate that feeder-free iPS cells are generated using FN-coated plates.

3.3.1.2 LN (*Laminin*)

LN is the primary ECM protein that appeared in 2- to 4-cell-stage embryos and is the main molecule of ECMs on the basal laminae in vertebrates.⁹³ LN is a member of the heterotrimeric glycoproteins, which consists of γ , α , and β chains where LN has 15 different combinations in human tissue.⁵³ LN-511 has the $\gamma 1$, $\beta 1$, and $\alpha 5$ chains and is commonly used as a coating ECM on the plates for hPS cell cultivation. LN-521, consisting of the $\alpha 5$, $\beta 2$, and $\gamma 1$ chains, has also recently been used for hPS cell culture (Table 3.1).^{20,53,99–101}

Several hES cell lines are known to display the LN-511 chain, whereas LN-332 does not exist in hES cells.^{53,99,102} Miyazaki and colleagues reported that hES cells primarily express integrin $\alpha 6 \beta 1$, which can bind extensively to LN-511/-521, LN-332, and LN-111 (Figure 3.4).⁹⁹ hES cells were found to grow on dishes immobilized with these three recombinant LNs in MEFs-CM (condition medium from MEFs) for several passages while hES cells maintain their pluripotency. It should be mentioned that MEFs-CM is obtained from the culture medium where MEFs are cultivated, which indicates the MEFs-CM is xeno-containing and an undefined medium, although the medium contains valuable growth factors and ECMs secreted by MEFs. These results indicated that recombinant LN-511/-521, LN-332, and LN-111 can support the proliferation of undifferentiated hES cells because of their high affinity of integrin $\alpha 6 \beta 1$.⁹⁹ However, this study was unfortunately done in xeno-containing cultivation medium, and the hES cells were only cultivated for a few passages. It is not clear whether the pluripotency of hES cells can be retained for longer passages (more than 10) in xeno-free cultivation media. However, Rodin and colleagues found that hES (HS401, HS207, and HS420) cells cultivated on LN-511-immobilized plates maintained pluripotency for over 20–25 passages with a normal karyotyping in xeno-free culture media of either H₃ or O₃ media.⁵³

3.3.1.3 VN (*Vitronectin*)

VN is a glycoprotein (75 kDa) composing approximately 460 amino acid residues.¹⁰³ Braam and colleagues reported hES cell adhesion on plates

coated with some ECMs (LN-111, VN, FN, and COL IV) at concentrations of $10.0\text{--}50.0\ \mu\text{g mL}^{-1}$ (Table 3.1).⁵⁰ The binding of hPS cells was mediated to VN by $\alpha\text{V}\beta 5$ integrins according to integrin-blocking measurements. $\alpha 5\beta 1$ integrin is responsible for the binding to FN and $\alpha 6\beta 1$ integrin is responsible for the binding to LN-111 as well as LN-332 and LN-511 (Figure 3.4).^{50,99} Only the recombinant VN-immobilized plates maintained the pluripotency and self-renewal of hES cells for at least 8–10 passages in mTeSR1 media in this study. Another investigator also suggested that VN-coated plates can maintain the pluripotency of hES cells (Table 3.1). Yap and colleagues investigated a threshold surface concentration of VN for the proliferation of hES (HES-3 and H1) cells maintaining their pluripotency.⁶² The successful expansion of hES cells was possible for over 20 passages on cell culture plates immobilized with $>5.0\ \mu\text{g mL}^{-1}$ human plasma VN in mTeSR1 media that indicates $250.0\ \text{ng cm}^{-2}$ of VN as evaluated from the Bradford analysis.⁶²

Meng and colleagues studied the synergistic influence of the cell cultivation material, media, and exogenous effects on the attachment and proliferation of hPS cells in xeno-free and defined cultivation conditions where hPS cells keep their pluripotency for few months.⁴⁷ These researchers noticed that hPS cell cultivation on VN-coated plates in TeSR2 media seemed to be optimal for long-term cultivation of three hES cell lines and two hiPS cell lines keeping their pluripotency, whereas hPS cell culture on FN-coated plates was not as optimal as most researchers have found recently.⁴⁷ VN (and LN), in concert with an appropriate cell cultivation media, should be considered as one of the optimum ECMs for maintaining proliferation of hPS cells.

3.3.2 hPS Cell Cultivation on Oligopeptide-immobilized Surfaces in 2D

Cultivation of hES cells on ECMs such as VN, LN-511 and LN-521, which are used to coat plates, maintains proliferation of hES cells keeping their pluripotency and the cell capability to induce differentiation into the cell derived from three germ layers for a long time in xeno-free cultivation media. Nonetheless, these ECMs are costly and are derived from animal sources, which leads to variability between batches.⁸³ Establishment of synthetic oligopeptides as active sites to bind and support the pluripotency of hPS cells is preferable. Melkounian and colleagues prepared synthetic cell culture dishes made up of polyacrylate conjugated to PAS (biologically active peptides) using EDC/NHS [1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/*N*-hydroxysuccinimide] reaction.⁶⁸ LN peptide (LM-PAS), short or long FN peptide (sFN-PAS or lFN-PAS, respectively), VN peptide (VN-PAS), and bone sialoprotein (BSP) peptide (BSP-PAS) are the biologically active peptides on PAS (Tables 3.2 and 3.5).⁶⁸ Only the BSP- and VN-derived peptides maintained adhesion and colony formation of hES

Table 3.5 Amino acid sequences of peptides for hPS cell cultivation.²⁴ Adapted from ref. 24 with permission from Elsevier, Copyright 2014.^a

Name (model ECM)	Amino acid sequence	Ref. (year)
BSP-PAS (BSP)	KGGNGEPRGDTYRAY	68 (2010)
VN-PAS (VN)	KGGPQVTRGDVFTMP	68 (2010)
sFN-PAS (FN)	GRGDSPK	68 (2010)
IFN-PAS (FN)	KGGAVTGRGDSPASS	68 (2010)
LN-PAS (LN)	KYGAASIKVAVSADR	68 (2010)
Cyclic RGD	GACRGDCLGA	78 (2010)
Oligo-HBP1 (VN)	GKKQRFRRNRKG	67 (2012)
Oligo-HBP2 (BSP)	FHRRIKA	67 (2010)
Oligo-HBP3 (FN)	GWQPPARARI	67 (2010)
PDL	-(Lysine)- _n	47 (2012)
Pronectin F (FN)	[(GAGAGS) ₉ GAAVTRGDSPASAAGY] ₁₂	83 (2012)

^aBSP, bone sialoprotein; FN, fibronectin; HBP, heparin-binding peptide; LN, laminin; PDL, poly-D-lysine; VN, vitronectin.

(H7 and H1) cells. This research was the first to show hES cell pluripotency where hES cells were cultivated on oligopeptide-immobilized plates in defined X-VIVO media. It should be noted that all of the peptides prepared in this study included the cell-binding peptides of arginine-glycine-aspartic acid (RGD), which indicates that the RGD peptide alone is insufficient for hES cells to adhere and keep their pluripotency.⁶⁸ The surface concentration of the oligopeptide is also critical, and in order to support the hES cell colony on the peptide surfaces, a concentration of BSP peptide >0.50 mM is necessary.⁶⁸ hES (H7) cells have been cultivated on VN-PAS and BSP-PAS surfaces for over 10 passages and supported self-renewal abilities with a normal karyotyping in several characterized media (TeSR2, mTeSR1, and X-VIVO 10).⁶⁸

The cyclic RGD peptide CRGDC has 10-fold higher strength than the linear RGD (*e.g.*, GRGDSP) peptide to hold together $\alpha 5\beta 1$, $\alpha V\beta 5$, and $\alpha V\beta 3$ integrins where the $\alpha 5\beta 1$ and $\alpha V\beta 5$ integrins are known to express in hES cells.^{50,67} Therefore, Kolhar and colleagues prepared dishes conjugated with cyclic RGD peptide (GACRGDCLGA) where the cyclic RGD peptide was bound to amine-immobilized tissue cultivation dishes *via* a bifunctional linker that reacted with amine groups on the cultivation dishes and a thiol of the cyclic RGD peptide (Figure 3.5).⁷⁸ The cyclic RGD peptide concentration of 10.0–30.0 fmol of peptides per cm² was found on the dishes from the surface concentration evaluation. The dishes conjugated with cyclic RGD peptides maintained the proliferation of hES (H14 and H9) cells keeping the pluripotency in MEFs-CM for 10–12 passages.⁷⁸ The differentiation ability of hES cells into three germ layer cells was evaluated by EB generation after 10 passages. The dishes grafted with cyclic RGD peptide, which have optimal stiffness, seem to promote the cultivation of hES cells for long periods, such as 10–30 passages.

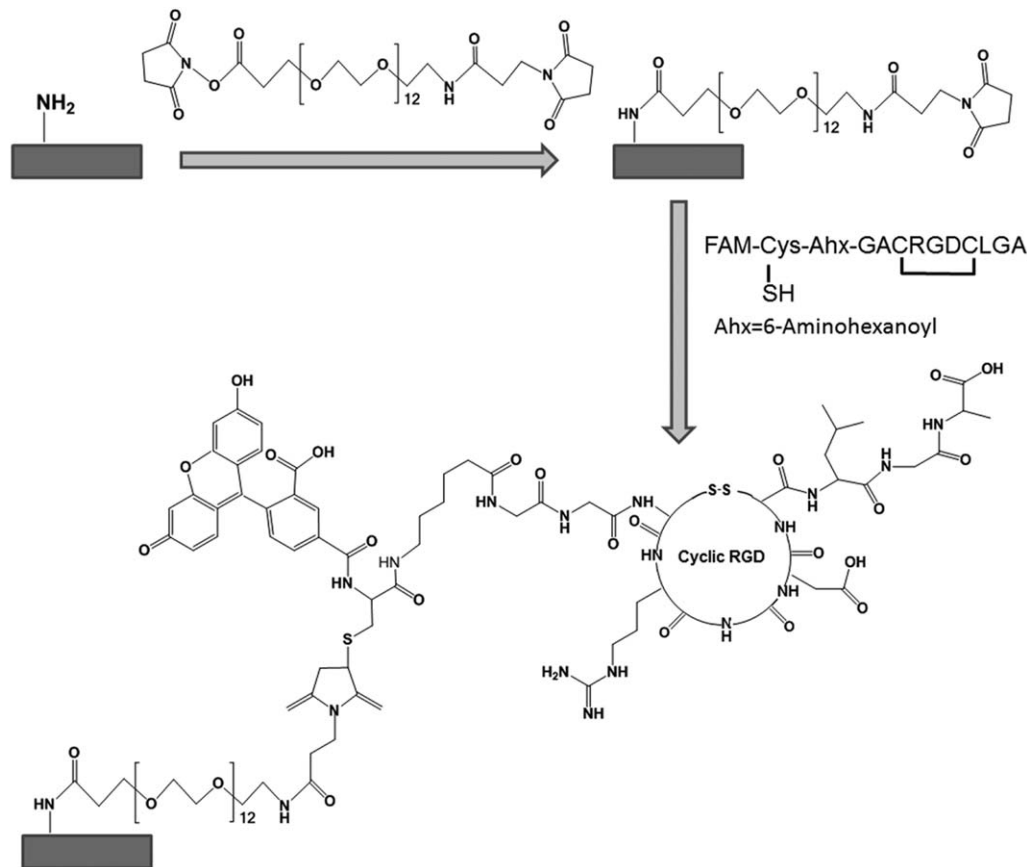


Figure 3.5 Preparation of dishes grafted with a cyclic RGD peptide (GACRGDCLGA). The cyclic RGD peptide was immobilized on amine-modified dishes *via* a bifunctional linker that reacts with a thiol of the peptide and an amine on the cell culture dishes.⁷⁸ Adapted from ref. 78 with permission from Elsevier, Copyright 2010.

Miyazaki and his colleagues described effective adhesion cultivation of hES cells using laminin fragment (iMax-511) when there is no pre-coating on cell culture plates.⁵⁸ Normally, cell culture substrates should be pre-coated (immobilized) with the whole or piece of ECM proteins and ECM-derived oligopeptides for proliferation of hPS cells.

However, they found that the simple addition of the laminin fragment (iMax-511) to a cell suspension during passaging promoted the adhesion of hPS cells onto cultivation dishes, which were uncoated with any ECM or ECM-derived oligopeptides.⁵⁸ Furthermore, the addition of laminin fragments maintained significant adhesion of hPS cells and maximum adhesion was observed at a much lower concentration than the concentration of laminin fragments used for the pre-coating of the cell culture dishes.⁵⁸ Strikingly, hES cells seeded with uncoated laminin fragments proliferated significantly without any cell detachment and supported their pluripotent state for over 10–12 passages. Other cultivation substrates using full-length laminin or vitronectin were also evaluated for hES cell adhesion in the uncoated manner. However, laminin is the only ECM that has the potential for expansion of hES cells in the uncoated manner.⁵⁸ This method can also contribute to hPS cell expansion and accelerate the improvement of stem cell therapy using hPS cells widely in microcarrier cultivation systems.

Klim and colleagues developed a well-characterized surface array of self-assembled monolayers made of alkanethiol conjugates with peptides to verify optimal sequences of oligopeptides, which maintain the pluripotency and proliferation of hPS cells (Table 3.2).⁶⁷ Eighteen bioactive oligopeptides were detected; the oligopeptides were selected from BMP-2, Dll-1, NCAM, FN, LN, VN, BSP, annexin, E-cadherin, and phage display libraries. The oligopeptides include the binding sites of polysaccharides and the integrin ligand RGD (Table 3.5).⁶⁷ The surfaces displaying heparin-binding oligopeptides (GWQPPARARI from FN, FHRRIKA from BSP, and GKKQRFRHRNRKG from VN) greatly support hES (H9) cell adhesion and maintain hES cell proliferation in mTeSR1 media with Y-27632 (ROCK inhibitor) where ROCK inhibitor is typically used to support pluripotency of hPS cells in single cell morphologies. In contrast, the typical integrin sequence of KGRGDS could not support pluripotency of hES cells extensively.⁶⁷ A defined glycosaminoglycan-binding substratum for human pluripotent stem cells, hES (DF19-97T, H14, H13, and H9) cell lines and hiPS (IMR-90) cells, cultivated on plates immobilized with GKKQRFRHRNRKG oligopeptide *via* biotin–streptavidin reaction in the medium including Y-27632 could proliferate and exhibited pluripotency and long-term self-renewal (up to 3–4 months, 16–18 passages) with normal karyotyping. These hPS cells could differentiate into cells derived from three germ layers after 3–4 months of cultivation. The hES cells were also cultivated on plates immobilized with cyclic RGD and GKKQRFRHRNRKG peptides for 1–2 months in the absence of Y-27632 in mTeSR1 media, and

hES cells were found to keep their pluripotency and self-renewal capacity with normal karyotyping.⁶⁷

Park and colleagues prepared polydopamine conjugated with bioactive oligopeptide as a coating molecule on different biomaterials based on bioinspiration from the strong adhesive characteristics of mussels.⁷⁰ The adhesion is based on repeating units of L-dopa (3,4-dihydroxy-L-phenylalanine) and lysine residues in the mussel adhesive pads of *Mytilus edulis* foot protein-5. The hES and hiPS cells could be extensively cultivated on the dishes immobilized with peptide-conjugated polydopamine, where the peptide sequence was obtained from vitronectin without or with cysteine residues to create dual or single chains. Dishes immobilized with peptide-conjugated polydopamine could significantly decrease elastic modulus.⁷⁰ The hiPS cells cultured on dishes immobilized with dual-chain peptide-conjugated polydopamine showed the focal adhesion protein vinculin and could organize the cytoskeletal element of F-actin, which leads to higher colony adhesion of hiPS cells, which is compared to colony adhesion on a single chain of peptides. The hES and hiPS cells could be cultured on the dishes immobilized with peptide-conjugated polydopamine for 10–15 passages in feeder-free conditions.⁷⁰ However, long-term cultivation of hES or hiPS cells could not be evaluated on the dishes immobilized with peptide-conjugated polydopamine in xeno-free conditions. Moreover, the impact of elasticity of cell cultivation biomaterial on hES and hiPS cell cultivation was not examined in this study.

Therefore, Higuchi and colleagues investigated hPS cell cultivation on materials having several elasticities, which were immobilized with nano-segments derived from vitronectin.⁷¹ They developed the dishes coated with polyvinylalcohol (PVA) copolymer hydrogels immobilized with a VN-derived peptide (KGGPQVTRGDVFTMP) with elasticities ranging from 10 to 30 kPa storage moduli by adjusting the crosslinking time (Figure 3.6).⁷¹ The hPS cells cultivated on the hardest biomaterials (30 kPa) were going to induce differentiation after 5 days of cultivation, and moreover the hPS cells cultivated on the appropriate elastic substrates (24–26 kPa) kept their pluripotent state for more than 20–30 passages under xeno-free culture (Figure 3.7).⁷¹ The results suggest that cell cultivation biomaterials with appropriate elasticity can retain the pluripotent state of hPS cells in cultivation.

Molecular configurations of peptide-grafted PVA hydrogels with appropriate elasticity were developed by Higuchi and colleagues, where the PVA hydrogels have branched-type chains, dual chains with joint segments, single chains with joint segments, and single chains (Figure 3.8).⁷⁶ Peptide sequences were chosen from glycosaminoglycan- and integrin-binding sites of ECMs. The hydrogels immobilized with VN-derived peptides with a dual chain and a joint segment, which has a storage modulus of 24–26 kPa, maintained the long-term cultivation of hiPS or hES cells for more than 10–12 passages.⁷⁶ The joint segment and/or dual chain having cell adhesive sites on the hydrogels promoted the expansion of hiPS and hES cells with pluripotency.

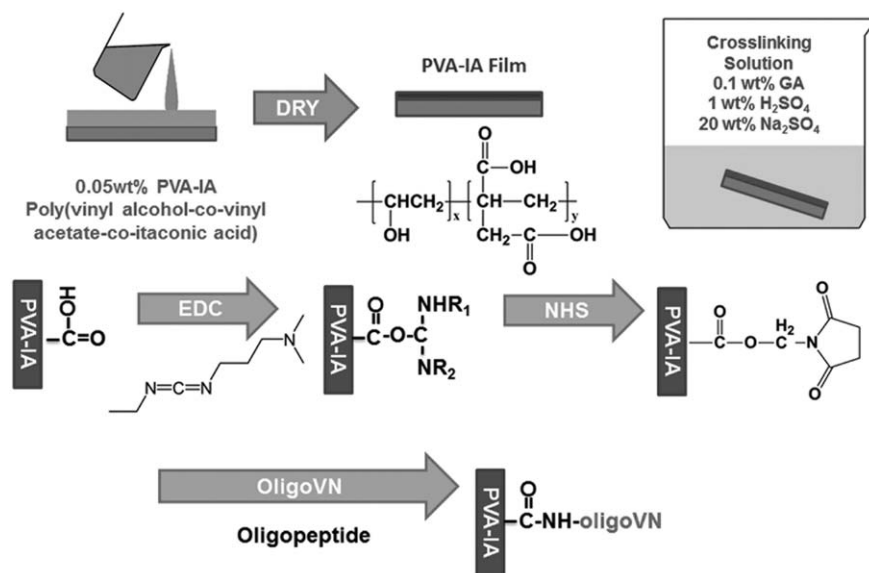
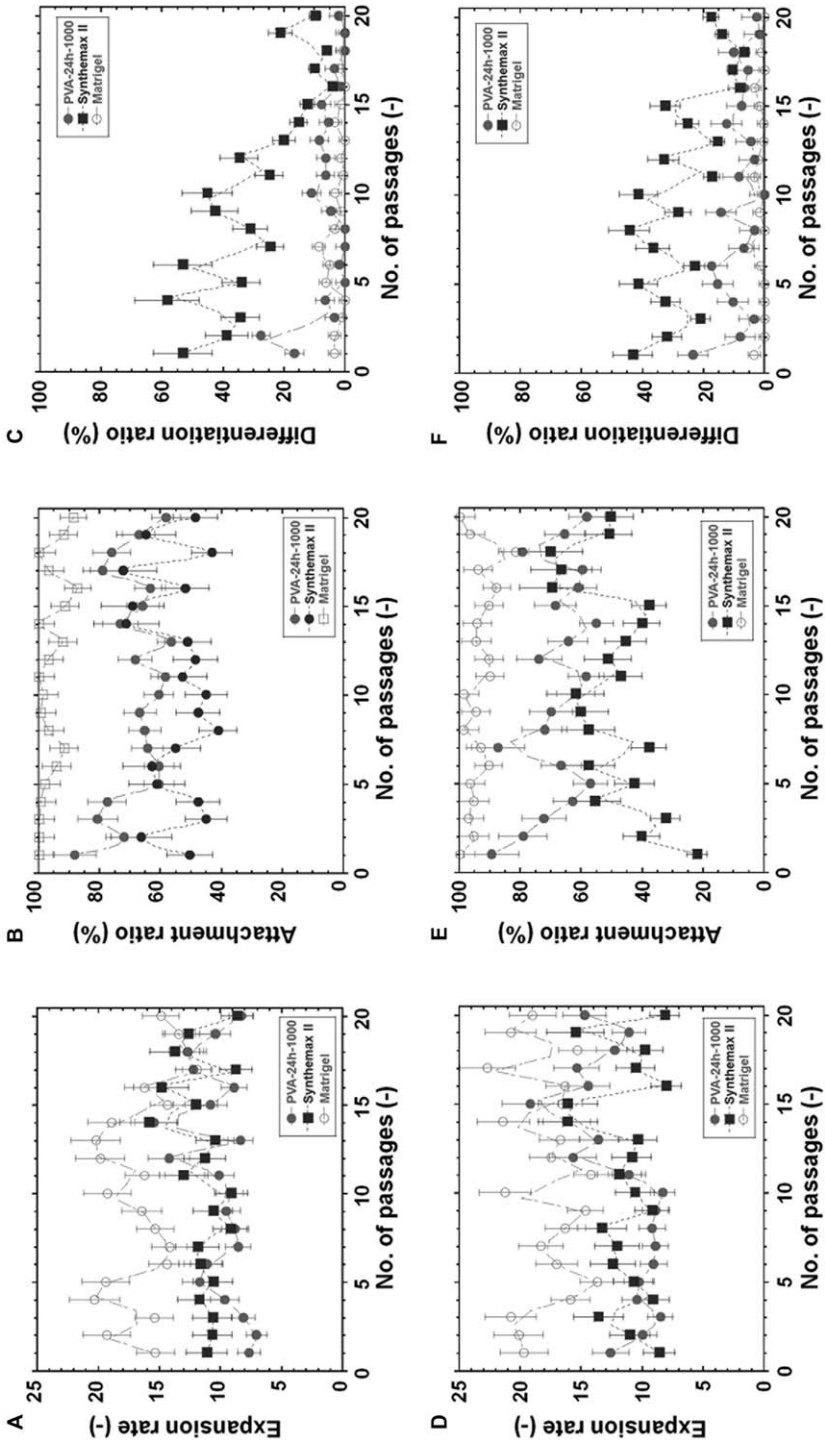


Figure 3.6 Synthesis and evaluation of PVA hydrogel immobilized with oligoVN. Chemical reaction for PVA hydrogels immobilized with oligoVN.⁷¹ Reproduced from ref. 71, <https://doi.org/10.1038/srep18136>, under the terms of the CC BY 4.0 license, <https://creativecommons.org/licenses/by/4.0/>.

These results indicate that surface density of cell-binding sites of ECM-derived peptides and molecular design of hydrogels are key points for facilitating the expansion culture of hiPS and hES cells to maintain the long-term pluripotent state of the cells in xeno-free cultivation conditions. Optimal oligopeptide-immobilized dishes can maintain hPS cells in a xeno-free and defined media and are preferable to ECM-immobilized plates because of their completely synthetic properties.

3.3.3 hPS Cell Cultivation on a Recombinant E-cadherin Surface in 2D

E-cadherin is a Ca²⁺-dependent cell-cell attachment molecule,¹⁰⁴ which is critical for colony formation and intercellular adhesion of hPS cells.^{93,105} hPS cells show high expression of E-cadherin. Nagaoka and colleagues designed fusion proteins composed of the Fc domain of immunoglobulin (IgG) and the extracellular domain of E-cadherin (E-cad-Fc) (Figure 3.9). They studied hES cell cultivation on the dishes coated with recombinant E-cadherin in both mTeSR1 and MEFs-CM media^{84,93} (Table 3.2).²⁵ The hES cells cultivated in this process maintained pluripotency for >34–36 passages and induced differentiation into the cells derived from three germ layers.

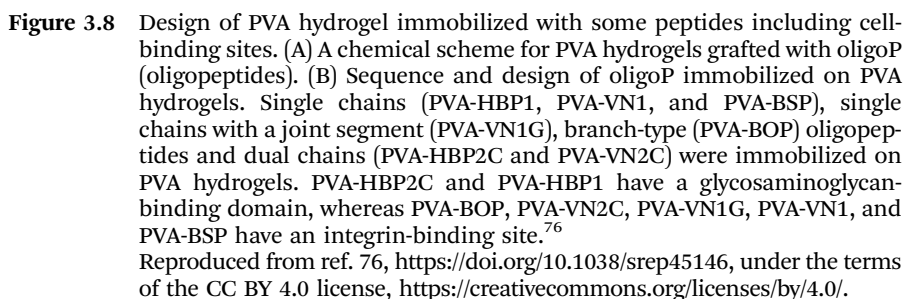


Integrin-mediated cell-ECMs adhesion are considered critical to maintain viability and pluripotency of hPS cells. As seen in previous sections, many studies have been devoted to finding optimal ECMs and ECM-derived oligopeptides that can support the pluripotency of hPS cells *via* the integrin binding among hPS cells and ECMs. ECM-integrin interactions promote activation of ILK (integrin-linked kinase) and/or FAK (focal adhesion kinase) signaling pathways and the MAPK and PI3K/Akt pathways.¹⁰⁶ Furthermore, the E-cadherin-mediated attachment of hPS cells is interacted with β -catenin signaling and induces the stimulation of PI3K/Akt signaling (Figures 3.4 and 3.9).^{84,93,107} The Akt signaling pathways are found to be extremely valuable in maintaining the undifferentiated state of hPS cells.¹⁰⁸ It is reported that *trans*-homodimerization between the E-cadherin site of recombinant E-cadherin on cell cultivation plates and E-cadherin on hPS cells maintains the pluripotent state of hPS cells through the activation of the PI3K/Akt signaling pathway.⁸⁴ The recombinant E-cadherin, which is used as a coating on the plates for hPS cell cultivation, is commercially available (StemAdhere™).⁸⁵

3.3.4 hPS Cell Cultivation on Biomaterials Immobilized with Polysaccharide in 2D

Some polysaccharides, *e.g.*, alginate and hyaluronan, were evaluated as potential materials for hPS cell cultivation.^{86,109–111} Polysaccharides play a valuable part in controlling hPS cell adhesion and proliferation.⁶⁷ For example, hyaluronan hydrogels may offer an appropriate physiological environment for proliferation of hPS cells because the feeder cells, which maintain the pluripotency and growth of hPS cells, secrete an abundance of hyaluronic acids in addition to a large amount of ECMs.^{109,112}

Figure 3.7 Long-term cultivation of hPS cells on PVA-oligoVN hydrogels having an optimal elasticity under xeno-free cultivation conditions. (A) Expansion rate of hES (WA09) cells on Matrigel (open circle), Synthemax II dishes (closed square), and PVA-24h-1000 dishes (closed circle) for 20 passages. (B) Attachment ratio of hES (WA09) cells on Matrigel (open square), Synthemax II dishes (closed square), and PVA-24h-1000 dishes (dark circle) for 20 passages. (C) Differentiation ratio of hES (WA09) cells on Matrigel (open circle), Synthemax II dishes (closed square), and PVA-24h-1000 dishes (closed circle) for 20 passages. (D) Expansion rate of hiPS (HPS0077) cells on Matrigel (open circle), Synthemax II dishes (closed square), and PVA-24h-1000 dishes (closed circle) for 20 passages. (E) Attachment ratio of hiPS (HPS0077) cells on Matrigel (open circle), Synthemax II dishes (closed square), and PVA-24h-1000 dishes (closed circle) for 20 passages. (F) Differentiation ratio of hiPS (HPS0077) cells on Matrigel (open circle), Synthemax II dishes (closed square), and PVA-24h-1000 dishes (closed circle) for 20 passages.⁷¹ Reproduced from ref. 71, <https://doi.org/10.1038/srep18136>, under the terms of the CC BY 4.0 license, <https://creativecommons.org/licenses/by/4.0/>.



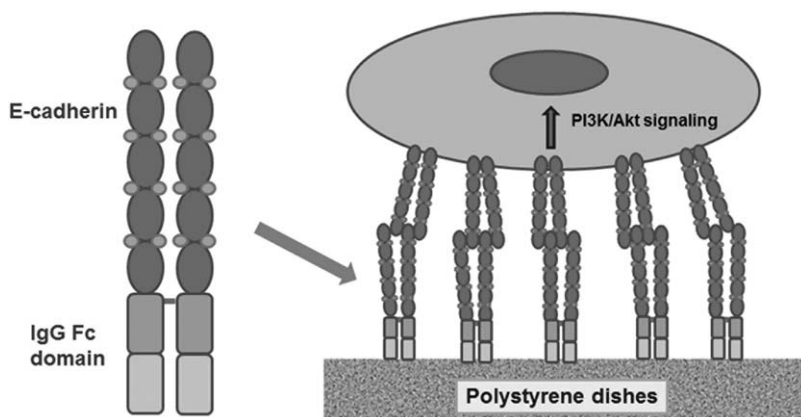


Figure 3.9 Models of hES cell attachment on an E-cadherin chimera-coated dish. The E-cadherin-mediated attachment of hES cells is generally correlated with β -catenin signaling, which activates PI3K/Akt signaling.²⁴

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Liu and colleagues developed hyaluronan-based hydrogels using poly(ethylene glycol) diacrylate, thiol-modified gelatin, and thiol-modified hyaluronan both without and with FN.¹⁰⁹ The growth speed of hiPS (HDFa-YK26) cells on the hyaluronan-based hydrogel (without and with FN) in MEFs-CM were found to be similar to the growth speed of hiPS cells cultured on Matrigels or MEFs for 10–12 passages. Furthermore, hiPS cells retained their pluripotency, their ability to induce differentiation into the cells derived from three germ layers *in vitro* (EBs formation assay), and their stable karyotyping.¹⁰⁹ hiPS cells were cultivated on a hyaluronan-based hydrogel containing FN but not on a hyaluronan-based hydrogel without FN in chemically defined media. Furthermore, the long-term cultivation of hiPS cells on a hyaluronan-based hydrogel having FN was found not to be adequate because of the mechanical weakness of the hyaluronan-based hydrogel having FN.¹⁰⁹

Compared with 2D cultivation, 3D cultivation more accurately mimics the *in vivo* niche with essential cell–matrix and cell–cell interactions. The difficult point with most 3D cultivation systems is that harvesting of proliferated hPS cells in 3D scaffolds and/or hydrogels is troublesome. Lu and colleagues prepared an ion complex microfiber scaffold consisting of cationic chitosan and anionic alginate; these polysaccharides can be enzymatically digested using chitinase and alginate lysate, respectively.⁸⁶ hES cell-embedded microfibers could be made by a spinning method from the border between an alginate and a chitosan solution including hPS cells, followed by dipping in a CaCl_2 solution for alginate crosslinking (Table 3.2, Figure 3.10).⁸⁶ Some cell lines of hPS cells could be embedded into the 3D microfiber system, and the cells were proliferated for 8–10 passages in

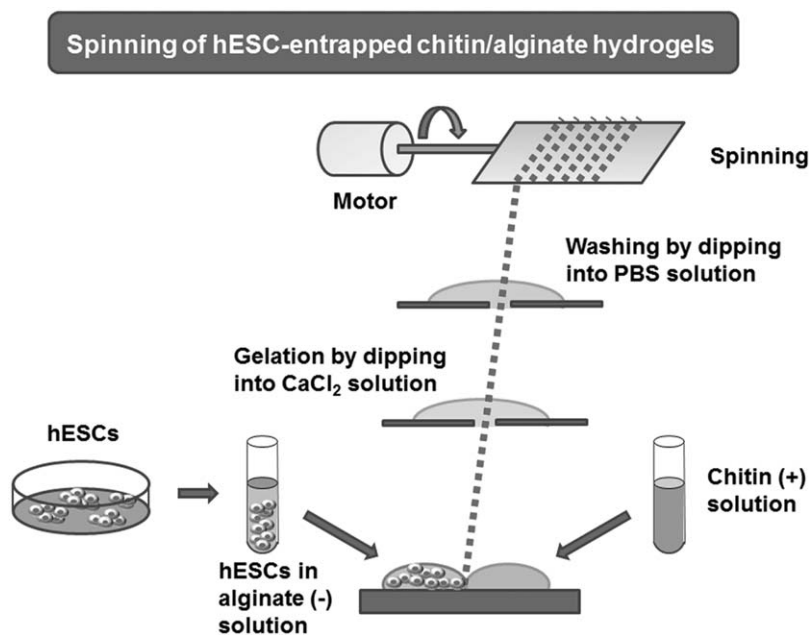


Figure 3.10 Spinning of hES cells entrapped in alginate/chitosan ion complex hydrogel. Microfibers were generated from the interface between the alginate solution and chitosan solution including hPS cells, followed by immersion in a CaCl₂ solution for alginate cross-linking (coagulation) and subsequently washing.⁸⁶ Adapted from ref. 86 with permission from Elsevier, Copyright 2012.

mTeSR1 media while keeping their pluripotent state and the ability to induce differentiation into the cells derived from three germ layers while maintaining reliable karyotyping. The 3D microfiber is able to encapsulate hPS cells at a high inoculating concentration (10 million cells per mL) while retaining good viability. The cell density was found to be almost 50 times higher than that used in a conventional 2D culture system.⁸⁶ The harvesting of hPS cells could be performed by injecting the 3D microfibers into alginate lysate and chitinase for 10–12 minutes. The hPS cells recovered from the 3D microfibers maintained good pluripotency and cell viability. Furthermore, the hPS cells entrapped in 3D microfibers could be directly cryopreserved, and more than 70–75% of the hPS cells were found to be viable after thawing in mTeSR1 media including Y-27632 (ROCK inhibitor). The recovery ratio was found to be over 16-fold higher than the recovery ratio found in hPS cells cryopreserved processing in the conventional protocol.⁸⁶ This is explained by the fact that the 3D microfiber systems are not necessary to detach the cells from cell culture biomaterials before cryopreservation.

3.3.5 hPS Cell Cultivation on Synthetic Biomaterials in 2D

The cultivation of hPS cells on chemically defined biomaterials prepared from the monomer synthesis negates the variables related to natural protein coatings or feeder cells that may produce batch-to-batch irregularities and lead to biosafety problems.²⁷ Typically, synthetic polymeric materials sustain only short-term proliferation or cultivation of hPS cells in media including xenobiotic products, such as MEFs-CM and FBS (fetal bovine serum). Recently, some synthetic polymeric biomaterials have been developed to hPS cell proliferation of hPS cells in chemically defined media or in xeno-free conditions (Table 3.2, Figure 3.11).^{87–91} In this section, we discuss types of synthetic polymeric materials, thermoresponsive synthetic polymeric materials and synthetic nanofibers that all maintain the long-term cultivation of hPS cells.

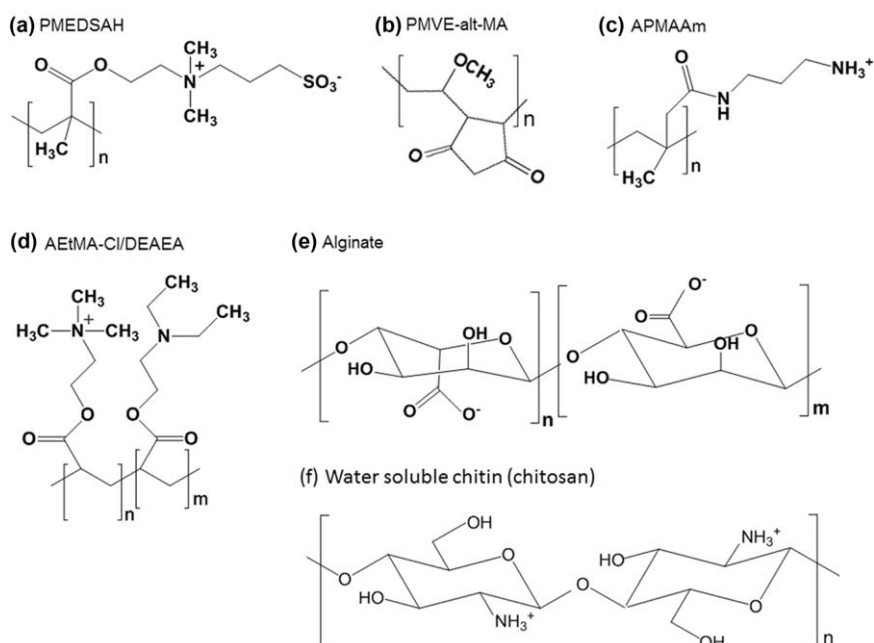


Figure 3.11 Scheme of the synthetic polymers used as scaffolds, hydrogels, and substrates for the expansion of hPS cells in defined media under xeno-free and feeder-free conditions. (a) PMEDSAH (poly(2-[methacryloyloxy]-ethyl dimethyl-[3-sulfopropyl] ammonium hydroxide)), (b) PMVE-alt-MA [poly(methyl vinyl ether-alt-maleic anhydride)], (c) APMAAm (aminopropylmethacrylamide), (d) AEtMA-Cl/DEAEA, copolymer of DEAEA [2-(diethylamino)ethyl acrylate] and AEtMA-Cl [2-(acryloyloxyethyl) trimethylammonium chloride], (e) alginate, and (f) water-soluble chitin (chitosan).²⁴

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3.3.5.1 Synthetic Polymeric Materials That Maintain the Long-term Cultivation of hPS Cells

A synthetic polymeric material, PMEDSAH (poly(2-[methacryloyloxy]ethyl dimethyl-[3-sulfopropyl] ammonium hydroxide)), was conjugated onto plates by means of surface-initiated graft polymerization (Table 3.2, Figure 3.12) that maintained the long-term cultivation of H9 hES cells with typical karyotyping far above the 10–12 passages in StemPro media.^{87,90} Moreover, hES cell adhesion was not found on PCBMA, poly(carboxybetaine methacrylate)-grafted plates, and the hES cells attached but spontaneously induced into differentiation during the initial two passages on PPEGMA, poly(poly[ethylene glycol] methyl ether methacrylate)-grafted, PHEMA, poly(2-hydroxyethyl methacrylate)-grafted, PMETAC, poly([2-(methacryloyloxy)ethyl])-grafted, and PSPMA, poly(3-sulfopropyl methacrylate)-grafted plates in MEFs-CM (Figure 3.12). Only PMEDSAH-grafted plates significantly maintained pluripotency of H9 cells in chemically defined media. However, some hES cell lines, for example BG01, did not support the pluripotent state for more than three passages on the PMEDSAH-immobilized plates in chemically defined media. However, the establishment of totally synthetic materials, for example PMEDSAH-grafted plates, is valuable for the maintenance and proliferation of hES cells in xeno-free and defined cultivation media.

The hydrogel interface of APMAAm (aminopropylmethacrylamide) could be appropriate for cultivating hES cells in mTeSR1 media (Table 3.2, Figure 3.11).⁸⁹ Healy and colleagues investigated whether hES cells (H9 and H1) were cultivated on APMAAm for more than 18–22 passages and retained the pluripotent state, similar to the data obtained for hES cells cultivated on Matrigels.⁸⁹ The authors evaluated the attachment of BSA and other proteins, for example TGF- β and FGF-2, onto an APMAAm hydrogel in mTeSR1 media and reported that the attachment of some appropriate proteins on the material would be important for hES cell attachment on the hydrogels.⁸⁹ Although BSA is typically used to inhibit the non-specific adhesion of other proteins on the material surface, the special configuration of BSA molecules and adhesion of other proteins in the media on APMAAm may generate a surface that maintains the long-term proliferation of hES cells. BSA, as well as other bioactive molecules, might promote the attachment of hPS cells by facilitating the production of ECMs from hPS cells and/or integrin expression by hPS cells when BSA is adhered on the cell culture biomaterials with an optimal configuration.

Different designs of synthetic polymeric materials are further reported to maintain the growth of hES cells with typical karyotyping. Brafman and colleagues established plates immobilized with approximately 100 types of synthetic polymeric materials for application in a high-throughput screening method.⁸⁸ From their evaluation of polymeric materials, PMVE-*alt*-MA [poly(methylvinyl ether-*alt*-maleic anhydride)] having a molecular weight of 1.3×10^6 Da was found to maintain the self-renewal and proliferation of hES

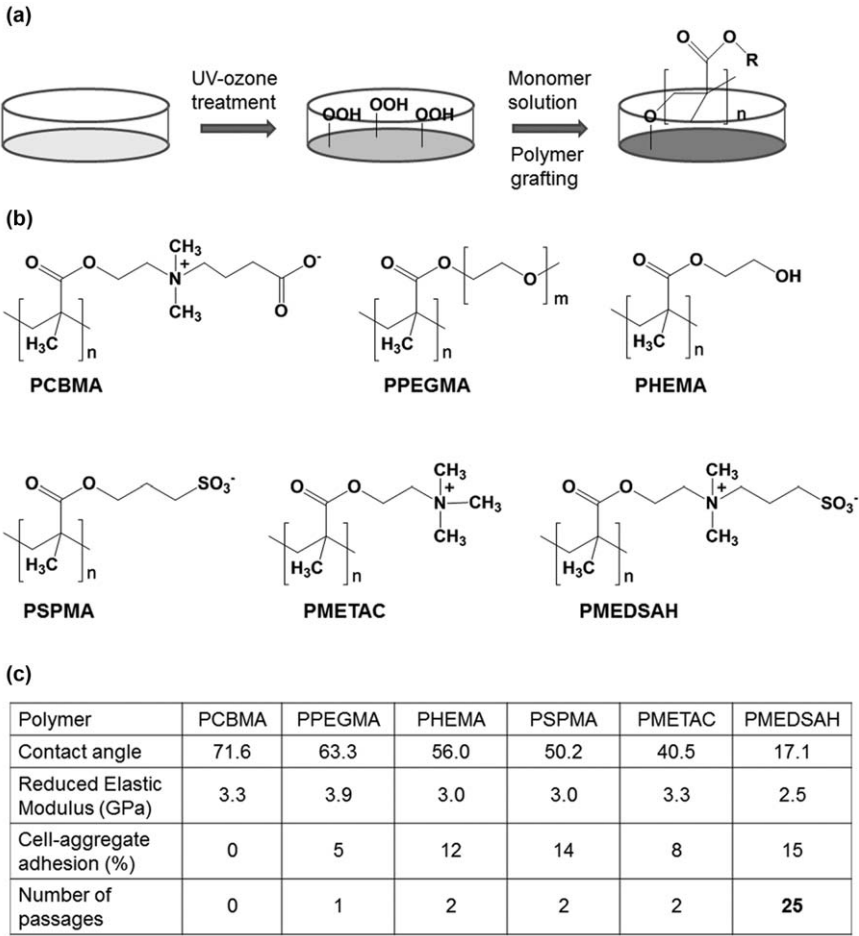


Figure 3.12 Long-term cultivation of hES (H9) cells on synthetic plates in MEF-CM. (a) Chemical scheme indicating the graft polymerization process used to synthesize the polymeric grafting. TCP dishes were activated by UV ozone at first; subsequently, methacrylate-based monomers were polymerized on TCP dishes. (b) Chemical scheme of the methacrylate-based monomers used for the polymeric grafting on TCP dishes. (c) The table shows the initial hES cell aggregate adhesion, reduced elastic modulus (GPa), contact angle and passage numbers obtained on each polymeric grafted dish.⁹⁰ Adapted from ref. 90 with permission from Elsevier, Copyright 2012.

(HUES9 and HUES1) cells for more than 5–6 passages in chemically defined media (Table 3.2, Figure 3.11). On the other hand, other typical polymeric materials maintained hES cell growth for only a short time.⁸⁸ ECMs are secreted by hES cells and play an important role in maintenance of pluripotency and cell adhesion and the integrins αV and $\alpha 5$ are primary integrins, which support hES cell adhesion and interaction with ECMs.

The expressing amount of endogenous ECMs and integrins in hES cells cultivated on PMVE-*alt*-MA were reported to be significantly higher than their rates in hES cells cultivated on Matrigel-coated plates.⁸⁸ Subsequently, PMVE-*alt*-MA seems to facilitate the expression of integrins and the secretion of ECMs by hES cells that maintain the growth of undifferentiated hES cells on PMVE-*alt*-MA-immobilized plates (Figure 3.2(d)).

3.3.5.2 Thermoresponsive Polymeric Materials That Maintain the Long-term Cultivation of hPS Cells

Chemical, enzymatic, and/or mechanical cell dissociation techniques are generally used as passaging methods for hPS cells. However, the techniques are not completely defined, which generates variability in the systems and could possibly lead to damaged cells. The enzymatic release of cells from cell culture plates sometimes produces the abnormalities of karyotyping.

Cells can be released from cell culture biomaterials by external stimulus, such as light irradiation or temperature decrease.^{113–116} In particular, the plates or micelles prepared with thermoresponsive polymeric materials having LCST (low critical solution temperature) can be employed for cell cultivation biomaterials, drug carriers, and other biochemical and medical applications.¹¹⁷ Zhang and colleagues investigated thermoresponsive polymeric materials using arrays consisting of over 600 different polymeric materials, located in quadruplicate, which were generated *in situ via* inkjet printing mixtures of 18 monomers with seven different ratios in the presence of the crosslinker *N,N'*-methylene-bis-acrylamide for hES (RH1) cell cultivation.⁹¹ The investigators noticed that a series of thermoresponsive hydrogels composed of AETMA-Cl [2-(acryloyloxyethyl) trimethylammonium chloride] and DEAEA [2-(diethylamino)ethyl acrylate], AETMA-Cl/DEAEA, maintained the long-term proliferation and pluripotent state of hES (H9 and RH1) cells in mTeSR1 media for more than 2–6 months (more than 18–25 passages) (Figure 3.11, Table 3.2).⁹¹ The hydrogel allowed reagent-free and gentle cell passage with >89–92% cell detachment *via* the transient change in temperature from 37 °C to 14–16 °C for 28–35 minutes. The biomaterials established in this work guide a scalable and flexible method for improvement of the safety and efficacy of hPS cell cultivation methods for clinical, engineering, and research use.⁹¹

Chen and his colleagues developed a method to generate and maintain hES cell aggregates in a 3D suspension culture by using thermoresponsive nanobridges, which were designed to be composed of poly(*N*-isopropylacrylamide) and recombinant fibronectin (PNIPAM-*b*-rFN).⁴² The property of the polymer used in the nanobridges enabled passaging and expansion of hESC cells (H9 and HES3) through a temperature change in combination with mechanically applied shear to dissociate aggregates.⁴² This method could eliminate the need for enzymes or small molecules for cell detachment and dissociation during passage of the cells. Using this

platform, hES cells could expand for three continuous passages, which produced high expression of key pluripotent markers (Oct4 and Nanog).

Higuchi and colleagues proposed a continuous harvest method for stem cells cultivated on thermoresponsive nanobrush surfaces.⁷⁵ In this method, stem cells were partially detached from the nanobrush surface by reducing the temperature of the cultivation medium below the LCST needed for thermoresponse (Figure 3.13A).⁷⁵ The detached stem cells were harvested by exchange into fresh cultivation medium. Following this, the remaining cells were continuously cultivated by expansion in fresh cultivation medium at

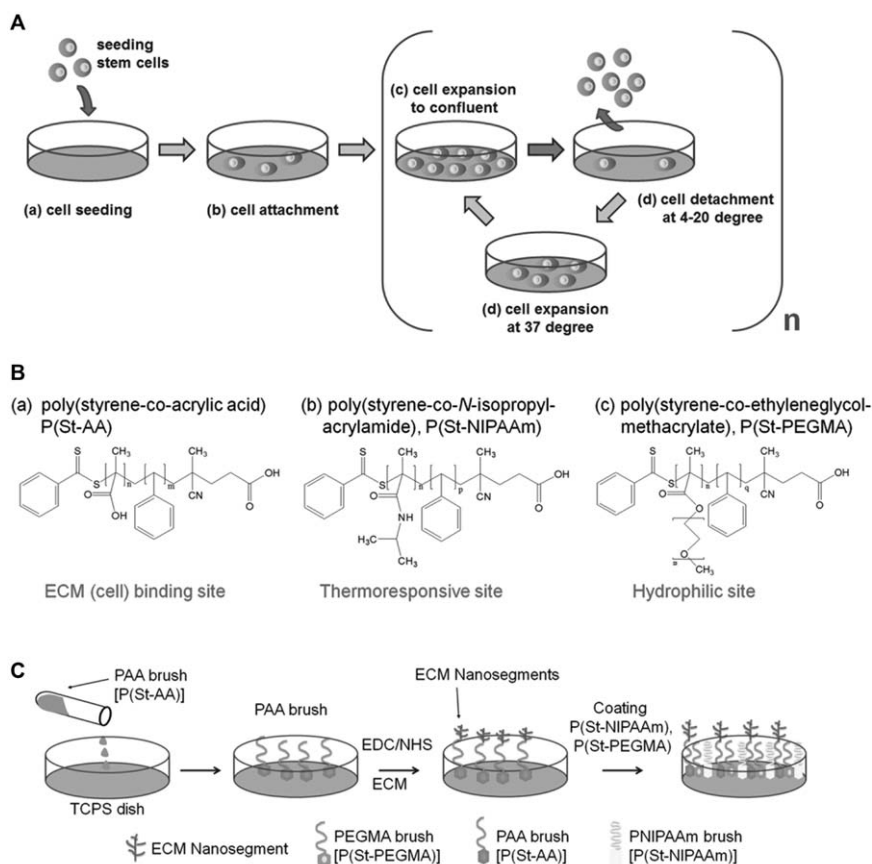
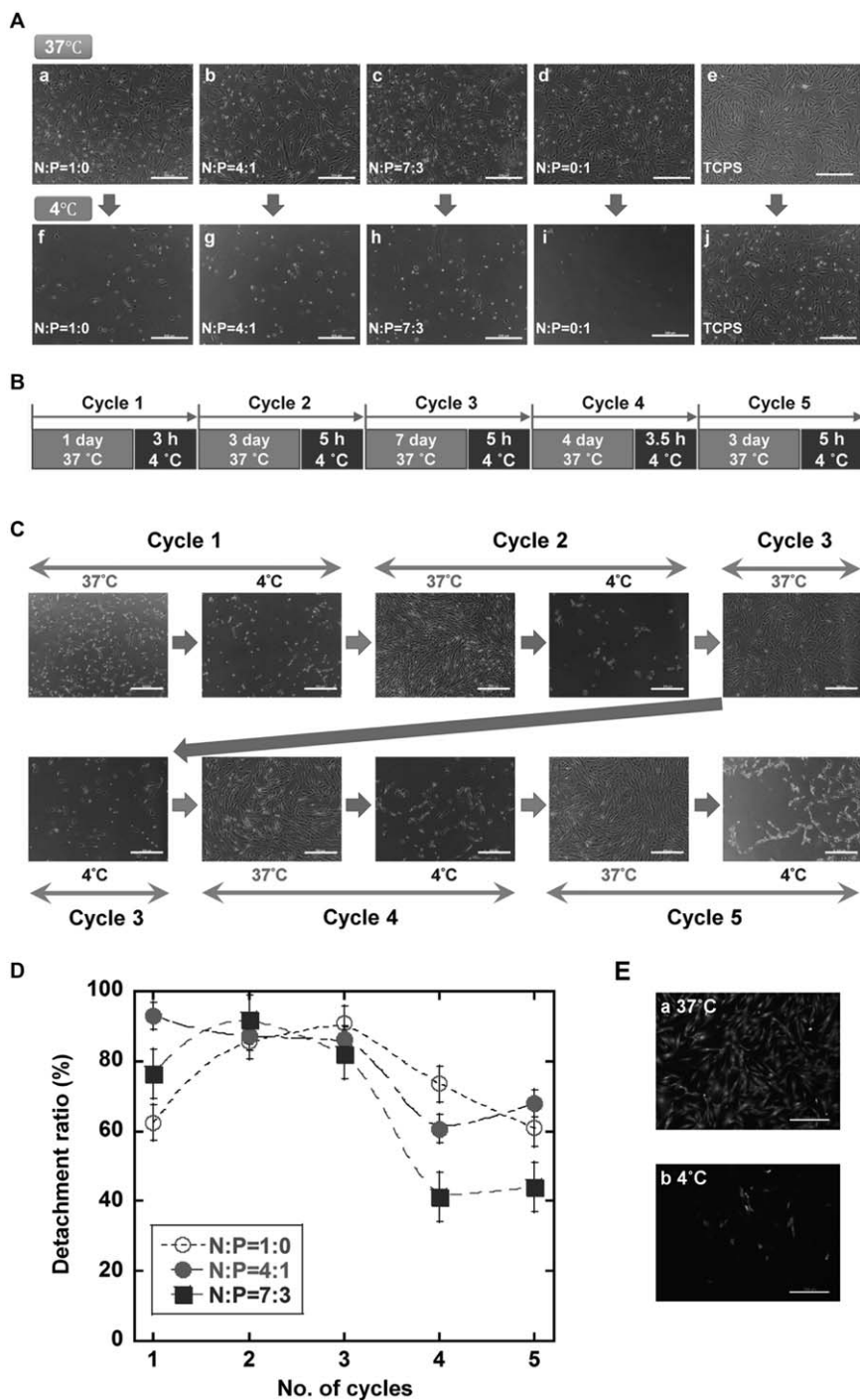


Figure 3.13 Preparation method of the thermoresponsive nanobrush surface. (A) Concept of continuous harvest of stem cells on the thermoresponsive nanobrush surface. (B) Chemical scheme of coating copolymers, poly(styrene-co-ethyleneglycolmethacrylate) (P[St-PEGMA]), poly(styrene-co-N-isopropylacrylamide) (P[St-NIPAAm]), and poly(styrene-co-acrylic acid) (P[St-AA]). (C) Preparation of the thermoresponsive nanobrush surface by coating P[St-AA] immobilized with P[St-PEGMA], P[St-NIPAAm], and oligoVN.⁷⁵

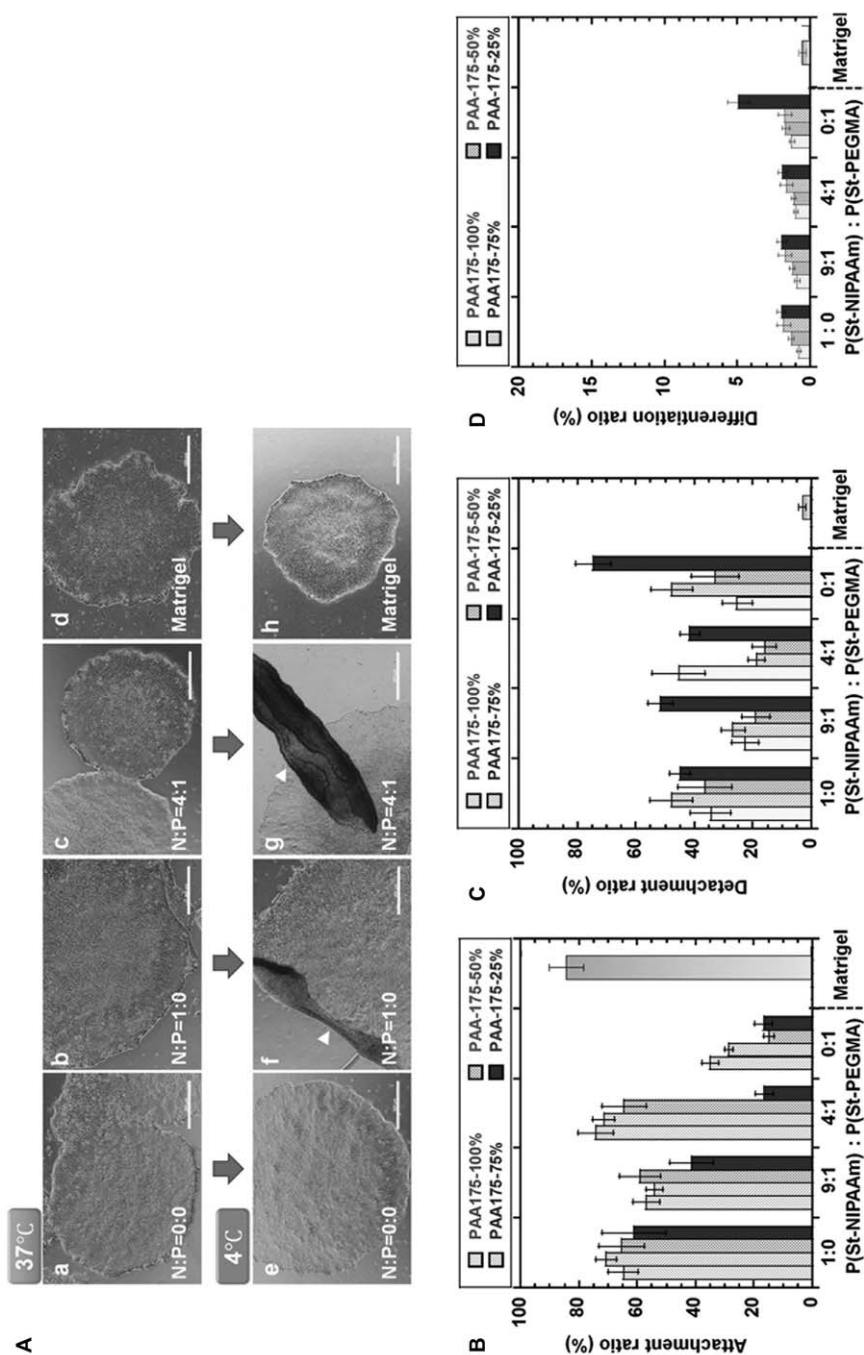
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37 °C. Thermoresponsive nanobrush surfaces were prepared by coating block copolymers containing polystyrene (for hydrophobic anchoring onto cultivation dishes) with three types of polymers: (1) polyacrylic acid with cell-binding oligopeptides (P[St-AA]-oligoVN), (2) thermoresponsive poly-*N*-isopropylacrylamide (P[St-NIPAAm]), and (3) hydrophilic poly(ethylene-glycol)methacrylate (P[St-PEGMA]) (Figure 3.13B and C).⁷⁵ The optimal coating durations and compositions for these copolymers to facilitate adequate attachment and detachment of hADS cells and hES cells were determined.

Five cycles of partial detachment and harvesting of hADS cells were performed from surfaces composed of 7:3, 4:1, and 1:0, 4:1 ratios of P[St-NIPAAm]:P[St-PEGMA] with 24–26% surface coverage of P[St-AA]-oligoVN (Figure 3.14A).⁷⁵ The timeline of temperature regulation is depicted in Figure 3.14B, and hADS cell pictures on several thermoresponsive dishes during continuous harvesting are shown in Figure 3.14C.⁷⁵ The hADS cells could be repeatedly recovered from the thermoresponsive dishes, whereas hADS cells did not detach from an untreated tissue culture polystyrene (TCP) surface. Figure 3.14D shows the hADS cell detachment ratio for every cycle of cell cultivation on the thermoresponsive dishes.⁷⁵ The detachment ratio could vary depending on the detachment processes. More than 58–62% detachment was found on the dishes coated with 4:1 and 1:0 ratios of P[St-NIPAAm]:P[St-PEGMA]. The hydrophilic nanobrush surfaces prepared by P[St-PEGMA] could not promote the repeated detachment (harvest) of hADS cells for over four cycles. The dead cells were not detected on the

Figure 3.14 hADS cell cultivation on the thermoresponsive nanobrush interface for the continuous harvest of stem cells. (A) The morphology of hADS cells cultivated on the surface coated with 24–26% coverage of P[St-AA]-oligoVN with 0:1 (d and i), 7:3 (c and h), 4:1 (b and g), and 1:0 (a and f) of P[St-NIPAAm]:P[St-PEGMA] and on TCP (e and j) after culture at 37 °C for 1 day (a–e) and subsequent culture at 4 °C for 6.0 h (f–j). The bar indicates 500 µm. (B) Timeline of temperature control of culture medium for continuous harvest of hADSCs on thermoresponsive nanobrush surface. (C) The morphology of hADS cells cultivated on the surface coated with 24–26% coverage of P[St-AA]-oligoVN with 4:1 of P[St-NIPAAm]:P[St-PEGMA] at continuous culture at 37 °C for 1–7 days and 4 °C for 3–5 h for five cycles. The bar indicates 500 µm. (D) Dependence of detachment ratio of hADS cells on the cycle of cell cultivation for partial detachment and harvest of hADS cells on the surface coated with 25% coverage of P[St-AA]-oligoVN with 7:3 (closed square), 4:1 (closed circle), and 1:0 (open circle) of P[St-NIPAAm]:P[St-PEGMA] at continuous culture at 37 °C for 1–7 days and 4 °C for 3–5 h for five cycles. (E) Dead/live staining of hADS cells after cultivation on the surface coated with 24–26% coverage of P[St-AA]-oligoVN with 4:1 of P[St-NIPAAm]:P[St-PEGMA] at 37 °C for 3 days (a) and subsequent culture at 4 °C for 5 h (b) at cycle 5 of the continuous harvest method. The bar indicates 500 µm.⁷⁵ Adapted from ref. 75 with permission from Elsevier, Copyright 2015.



thermoresponsive nanobrush dishes either after or before a single round of the detaching process (Figure 3.14E).⁷⁵ In particular, dead cells were not detected from flow cytometry analysis after five cycles of the partial detaching process.

Because hADS cells could be easily detached from thermoresponsive nanobrush dishes by lowering the temperature, and unwilling to use enzymes, Higuchi and colleagues examined hES (WA09) cell cultivation and removal from the thermoresponsive dishes.⁷⁵ In Figure 3.15A, the morphologies of hES cells adhered to the dishes immobilized with 4:1, 1:0, and 0:0 ratios of P[St-NIPAAm]:P[St-PEGMA] with 24–26% surface coverage of P[St-AA]-oligoVN after cultivation at 37.0 °C are given.⁷⁵ Cells cultured on Matrigel-coated dishes were used as standard experiments (Figure 3.15A). hES cell morphology following removal from the thermoresponsive dishes *via* exposure at 4–6 °C for 5.8–6.2 hours was investigated. hES cells could be easily cultivated on the thermoresponsive nanobrush dishes. When the cultivation temperature was reduced to 4–6 °C for 5.8–6.2 hours, hES cells were removed from the nanobrush dishes made of 4:1 and 1:0 ratios of P[St-NIPAAm]:P[St-PEGMA] with no pipetting procedures.⁷⁵ Consequently, when lowering the temperature on the Matrigel-coated dishes, hES cells were not removed from the Matrigel surface. Attachment ratios of hES cells following cultivation on dishes composed of 0:1, 4:1, 9:1, and 1:0 ratios of P[St-NIPAAm]:P[St-PEGMA] with 100% (PAA175-100), 75% (PAA175-75), 50% (PAA175-50), and 25% (PAA175-25) surface coverage of P[St-AA]-oligoVN at 37 °C are shown in Figure 3.15B, and hES cell detachment ratios are also given in Figure 3.15C.⁷⁵ The surface with 50–100% surface coverage of P[St-AA]-oligoVN appeared to be an excellent attachment ratio of hES cells (>54%) at 4:1, 9:1, and 1:0 ratios of P[St-NIPAAm]:P[St-PEGMA]. Consequently, the dishes with 24–26% surface coverage of P[St-AA]-oligoVN and

Figure 3.15 hESC culture and detachment on the thermoresponsive nanobrush interface. (A) The morphology of hES (WA09) cells cultured on the surface coated with 24–26% coverage of P[St-AA]-oligoVN with 4:1 (c and g), 1:0 (b and f), and 0:0 (a and e) of P[St-NIPAAm]:P[St-PEGMA] and on Matrigel (d and h) after culture at 37 °C for 5 days (a–d) and subsequent culture at 4 °C for 6 h (e–h). The arrowhead shows the detached hES cell colony. The bar indicates 500 μm. (B) Attachment ratio of hES (WA09) cells cultured on the surface coated with 100, 75, 50, and 24–26% coverage of P[St-AA]-oligoVN with 0:1, 4:1, 9:1, and 1:0 of P[St-NIPAAm]:P[St-PEGMA] and on Matrigel after culture at 37 °C for 5 days. (C) Detachment ratio of hES (WA09) cells cultured on the surface coated with 100, 75, 50, and 25% coverage of P[St-AA]-oligoVN with 0:1, 4:1, 9:1, and 1:0 of P[St-NIPAAm]:P[St-PEGMA] and on Matrigel after culture at 37 °C for 5 days and subsequent culture at 4 °C for 6 h. (D) Differentiation ratio of hES (WA09) cells cultured on dishes coated with 100, 75, 50, and 25% coverage of P[St-AA]-oligoVN with 0:1, 4:1, 9:1, and 1:0 of P[St-NIPAAm]:P[St-PEGMA] and on Matrigel after culture at 37 °C for 5 days.⁷⁵ Adapted from ref. 75 with permission from Elsevier, Copyright 2015.

0:1, 4:1, and 9:1 ratios of P[St-NIPAAm]:P[St-PEGMA] gave not so good attachment ratios of hES cells (<39%). hES cells showed a lower ability to attach to the thermoresponsive nanobrush dishes than hADS cells. The detachment ratio of hES cells was appreciably lower (<54%), except on the dishes with a 0:1 ratio of P[St-NIPAAm]:P[St-PEGMA] and 24–26% coverage of P[St-AA]-oligoVN.⁷⁵ This is explained as hES cell colonies had a better affinity for this appropriate dish than did hADS cells, because of the larger surface areas of hES cell colonies. Consequently, hES cell colonies were more difficult to remove from the surface than hADS cells when the P[St-NIPAAm] brush on the dishes was hydrated at 4–6 °C. In particular, heterogeneous adhesion of PNIPAAm brushes on the dishes significantly made variable hES cell detachment, which resulted in only small detachment of hES cell colonies.⁷⁵

This study described in detail how cultivation on thermoresponsive nanobrush dishes made it possible for hADS cells and hES cells to be continuously harvested for five and three cycles, respectively, when using a partial detachment protocol. Such continuous harvest of stem cells should reduce the equipment size and cost for stem cell cultivation as well as simplifying the cultivation process. These improvements should reduce the costs of therapies using hES cells as well as hiPS cells in the future.

3.3.5.3 Synthetic Microfibrous Scaffolds That Maintain the Long-term Cultivation of hPS Cells

Typical synthetic polymeric materials that maintain hPS cell proliferation include hydrogels, such as AEtMA-Cl/DEAEA,⁹¹ PMVE-*alt*-MA,⁸⁸ APMAAm,⁸⁹ and PMEDSAH,^{87,90} and the process is performed in combination with xeno-free and/or defined media (Table 3.2; Figure 3.11). The evaluation of systematic hydrogels will promote the future design of cell culture materials for the long-term proliferation of hPS cells. The development of chemically sustainable, controllable, and defined cell cultivation materials made of synthetic hydrogels on microfibers or plates is important for elucidating the mechanisms that regulate hPS cell behavior and for optimizing the parameters for the safe clinical use of hPS cells. Currently, the mechanism to maintain the pluripotency of hPS cells on these synthetic hydrogels is considered to be heparin mimicking characteristics, where the hydrogels significantly adsorb growth factors such as FGF-2 and TGF- β , which allows hPS cells to adhere to the hydrogels specifically *via* growth factor receptors on hPS cells.

Conventional hPS cell cultivation systems mainly involve 2D plates, which do not adequately mimic the complex 3D niches that cells inhabit during embryonic development.¹¹⁸ Therefore, current 2D cultivation of hPS cells *in vitro* may have restricted capability to replicate the 3D microenvironment *in vivo*. Carlson and colleagues reported valuable parameters of proliferation of hES (H9 and H1) cells on PDL (poly-D-lysine)-immobilized polymeric

materials with different conformations, which involved microfibrous scaffolds generated using the electrospun method, a macroporous scaffold (sponge) with a non-fibrous configuration, and a 2D polymeric film (Table 3.2).⁸² The base polymeric materials were pDTEC [poly(desamino-tyrosyltyrosine ethylestercarbonate)]. hES cells could not attach to both the 3D macroporous scaffolds and the 2D materials coated with PDL. The electrospinning microfibers coated with PDL allowed cell attachment and proliferation, and maintained the differentiation abilities of hES cells into three germ layer lineages for at least 2 weeks in mTeSR1 media.⁸² However, the long-term cultivation of hES cells is important in investigating whether microfibrous scaffolds coated with PDL are able to maintain pluripotency of hES cells for longer (*i.e.*, >10–30 passages). The authors noticed that hES cell attachment in microfibrous scaffolds generates increased confinement in the scaffolds that controls an enhancement of cell–cell contacts that leads to colony formation. Furthermore, the microfibrous scaffolds lead hES cells to secrete large amounts of ECMs, particularly those containing LN.⁸² hES cells carry the potential to create fibrous microenvironments using endogenous ECMs, and ECM deposition should be independently controlled, depending on the configuration of the cell cultivation materials. Within 3D fibrous materials, the increased local surface area relative to that of 2D plates and 3D macroporous scaffolds leads to the aggregation of hES cells during inoculation. With this aggregation, hES cells establish cell–cell contacts, and endogenously generated ECMs are able to coagulate, guiding the formation of pluripotent hES cell colonies.⁸² Although the simple method of hPS cell harvest from 3D cell cultivation materials could be improved, it seems that synthetic materials with appropriate 3D niches containing adhesive molecules such as oligopeptides, ECM, and PDL promote the configuration of pluripotent and self-renewing hES cell colonies, which support the ability to induce differentiation into the cells derived from three germ layers.

3.4 Three-dimensional Cultivation of hPS Cells on Biomaterials

Conventional 2D cultivation systems, as described in the previous sections, are easy to adapt for hPS cell cultivation in standard techniques and are important for evaluating the mechanism of the pluripotent maintenance of hPS cells cultivated on several materials. Furthermore, the morphologies of hPS cell colonies (*i.e.*, pluripotency of hPS cells) are identified by light microscopic detection *in situ*. The detachment of hPS cells from cell culture materials and the pick-up of pluripotent hPS cells from an hPS cell colony are easily carried out under light microscopic detection. However, the efficient and robust methods of generating hPS cells are hPS cell culture in 3D cultivation systems, because huge numbers of hPS cells are required in stem cell therapy. A minimum of 1×10^9 cells are typically needed to treat one

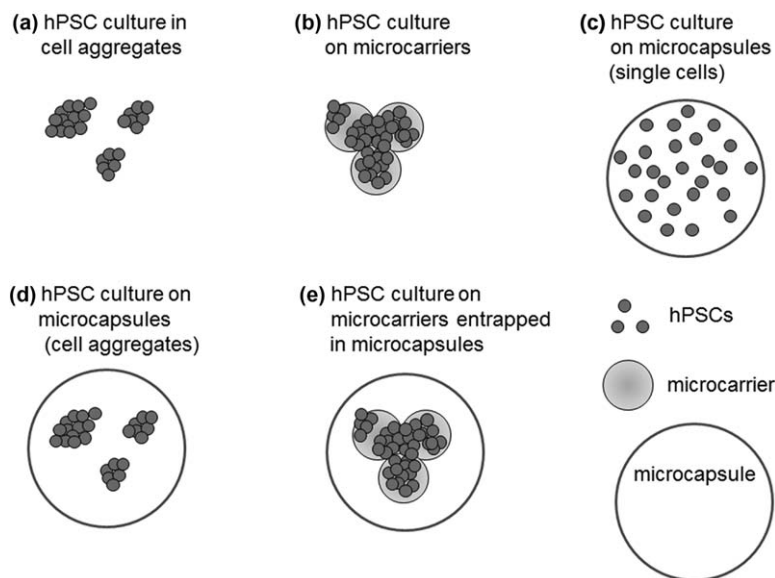


Figure 3.16 Schematic models of hPS cell cultivation in 3D. hPS cells are cultivated (a) in cell aggregate, (b) on microcarrier, (c) on microencapsulated single cell, (d) on microencapsulated cell aggregate, and (e) on microcarrier entrapped in microcapsule.¹²³ Reproduced from ref. 123, <https://doi.org/10.1371/journal.pone.0023212>, under the terms of the CC BY 4.0 license, <https://creativecommons.org/licenses/by/4.0/>.

patient in current stem cell trials, such as therapy to treat myocardial infarction. This number indicates that over 125 plates of 75 cm² T-flasks are necessary, assuming a confluent cultivation of hPS cells with 1.0×10^5 cells cm⁻².^{119–121}

Several 3D cultivation strategies for hPS cells were considered: (1) cell cultivation in a macroporous scaffold, (2) cell cultivation on nanofibers or microfibers, (3) cell aggregate (self-aggregated spheroid) cultivation, (4) cell cultivation on MC, and (5) microentrapped cell cultivation in suspended hydrogels (Figure 3.16).^{122,123}

Because of IL6RIL6 chimera (interleukin-6 receptor fusion to interleukin-6) or Y27632 (ROCK inhibitor), hPS cells can be cultivated on cell aggregates or in a single state.^{124–127} There are some studies reporting hES cell aggregate cultivation in suspension.^{122,124–128} However, these cultivation processes are beyond the scope of this book. Moreover, the cell aggregate cultivation of hPS cells in suspension was shown not to be as reproducible or efficient. In particular, this process requires the use of an expensive reagent, ROCK inhibitor, and a high concentration of FGF-2.¹²⁹ Hence, the MC suspension cultivation of hPS cells or the cultivation of microentrapped hPS cells in hydrogels appears to be more appropriate compared to a cell aggregate cultivation in suspension for 3D cultivation in a scalable and homogenous stirred 3D

cultivation process, although hPS cell harvest from a 3D culture system should be developed, such as using thermoresponsive polymers. These cultivation methods are discussed in the following sections.

3.4.1 The 3D Cultivation of hPS Cells on Microcarriers

hPS cells are sometimes cultivated on microcarriers (MCs) in suspension due to the high surface-to-volume ratio that is generated for MC cell cultivation systems. MCs are used for the scalable cultivation of anchorage-dependent cells, such as human hepatocytes and human retinal pigment epithelial cells, and for the co-cultivation of astrocytes and neurons.¹³⁰ MCs are generally composed of spherical particles made of various biomaterials such as polyester, glass, polystyrene (PS), and cellulose (CA), with a diameter around 90–270 μm .¹³⁰ Table 3.6 summarizes recent investigations into the cultivation of hPS cells on MCs.^{54,63,65,66,101,119,129–141}

MC systems have the flexibility of cell culture either on the surfaces of the MCs or within a macroporous scaffold. Phillips and colleagues studied the adhesion and proliferation of hES (ESI-017) cells on MCs composed of some polymeric materials with several configurations in MEFs-CM (Figure 3.17; Table 3.6).¹¹⁹ The MCs were used without MEF adhesion or coating by ECMs or Matrigels. The hES cells could adhere to the Hillex II MCs composed of the crosslinking PS carrying triethyl ammonium, which had a cationic charged surface. The hES cells also adhered to the Cultisphere with a cationic ionized surface. In contrast, hES cells did not attach to the Solohill plastic + or Solohill pronectin MCs or to the Cytodex 3 MCs composed of crosslinked dextran. Solohill plastic + is composed of crosslinked PS MCs having a cationic group, and Solohill pronectin includes pronectin-immobilized PS MCs.¹¹⁹ Pronectin is a peptide including the RGDS peptide and is prepared to mimic FN. Only small numbers of hES cells attached to the Cytodex 1 MCs that were composed of crosslinked dextran having a diethylaminoethyl site and a cationic group.¹¹⁹ Typically, the positive ionized surface of MCs such as Hillex II facilitates hES cell attachment on the surface from electrostatic interaction. This is because of the negatively charged surface of the cells in the resting state. Furthermore, other factors such as materials, shape, and the size of the MCs have an effect on the hES cell attachment on the MCs because, in this research, several MCs with a positively ionized surface could not facilitate hES cell attachment to the MCs (e.g., Cytodex 1).¹¹⁹ A COL immobilized on the MCs (Solohill COL) could not facilitate better hES cell attachment than the attachment using the cationic ionized microspheres (Hillex II). The MCs used in this research were not immobilized with VN, LN, or Matrigels; subsequently, the hES cells did not proliferate for more than 3–6 passages, and the expansion time depended on which cell detachment enzyme, trypsin or collagenase, was used.

hES cells could be cultivated on MCs without an ECM or Matrigel coating in some studies by other investigators.^{129,131,135} However, the hES cell cultivation times were found to be fewer than 6 passages and so most

Table 3.6 Study targeting proliferation of hPS cells cultivated on some microcarriers.²⁴ Adapted from ref. 24 with permission from Elsevier, Copyright 2014.

Cell line	Microcarrier name	Materials of microcarrier	Shape	Coating materials on microcarriers	Medium	Fold expansion (doubling time ^a)	Longest culture time	Reference (year)
hESCs (H9)	Cytodex 3	Crosslinked dextran with	Spherical, microporous	None	MEF-CM	6-fold for 4 days	14 days	131 (2009)
hESCs (H1, H9)	Cytodex 3	denatured collagen on surface		MEF or Matrigel	MEF-CM	3-fold in 60 h (DT = 35 h)	11 passages	130 (2009)
hESCs (SCED)	Cytodex 3			Matrigel	MEF-CM with Rock inhibitor	15-fold for 11 days	14 days	132 (2010)
hESCs (HES-2, HES-3)	Cytodex 1	Diethylaminoethyl (cationic) dextran	Spherical	Laminin or Matrigel	MEF-CM	20-fold for 7 days	10 passages	101 (2011)
hESCs (ESI-017, HUES9)	Hillex II	Crosslinked PSt modified with	Spherical	None	MEF-CM	3-fold for 5 days	6 passages	119 (2008)
hESCs (H9)	Hillex II	cationic trimethyl ammonium	Spherical	None	MEF-CM	DT = 30.3 h	264 h	129 (2013)
hESCs (HES-2, HES-3)	DE53	Diethylaminoethyl (cationic) cellulose	Microgranular cylindrical	Matrigel	mTeSR1, StemPro	10-fold for 7 days (DT = 21 h)	7 days	133 (2009)
hESCs (HES-3). hiPS (IMR90)	DE53		Microgranular cylindrical	Matrigel	mTeSR1	10–21-fold for 7 days (DT = 31–36 h)	10 passages	134 (2013)
hESCs (HES-2, HES-3)	DE53		Microgranular cylindrical	Laminin or Matrigel	MEF-CM	20-fold for 7 days	10 passages	101 (2011)
hESCs (HES-3, H7)	Not specified	Crosslinked PSt microcarrier	Spherical	LN or VN	StemPro	8.5-fold for 7 days (DT = 25 h)	20 passages	54 (2012)
hESCs (SHEF-3)	Cultispher-S	Crosslinked porcine gelatin	Spherical, macroporous	None	KO-DMEM with KSR, bFGF, periodically used Y-27632	5–10-fold for 7 days	2 passages	135 (2010)
hESCs (H1, H9)	Hyclone	Collagen-coated microcarriers	Spherical	Matrigel	MEF-CM	13-fold for 8 days (DT = 36.7 h)	12 days	136 (2009)

^aDT, doubling time.

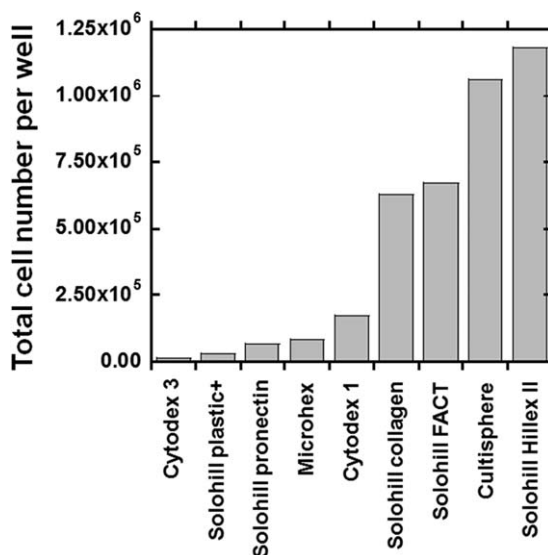


Figure 3.17 Growth and attachment of hES (ESI-017) cells on microcarriers. The number of attached ESI-017 cells cultivated on nine microcarriers in MEF-CM after a 72 h cultivation. The initial inoculation number was 1.0×10^6 cells.¹¹⁹ Adapted from ref. 119 with permission from Elsevier, Copyright 2008.

investigators^{54,101,130} use ECMs or Matrigels immobilized on the MCs to proliferate hPS cells and to support their pluripotency.

Nie and colleagues studied hES (H9 and H1) cell attachment and cell viability on some MCs in MEFs-CM after 5 days of cultivation; the MCs were composed of PS, gelatin, and crosslinked dextran, which had different surface charges and segments.¹³⁰ The MCs were coated with LN and FN to promote hES cell adhesion on the MC surface. A visual evaluation of hES cell adhesion and cell viability is shown in Table 3.7.¹³⁰ hES cell survival was not detected for some types of crosslinked PS MCs (PF102, PP102, P102, G102, C102, F102, and H11-921) immobilized with LN and FN. Crosslinked dextran MCs (Cytodex 3 and 1) immobilized with LN and FN facilitated hES cell attachment. In particular, the hES cells that were cultivated on crosslinked dextran MCs with denatured COL (Cytodex 3) and immobilized with LN and FN expressed good cell attachment on the MCs with excellent cell viability in MEFs-CM.¹³⁰

Chen and colleagues also studied the influence of several species of MCs on the pluripotency, cell proliferation, and adhesion efficiency of hES (HES-3 and HES-2) cells in MEFs-CM; the MCs were immobilized without and with Matrigels or ECMs (Table 3.6).¹⁰¹ The MCs in this study were (1) Tosho 10 PR and Tosho 65 PR (spherical hydroxylated methacrylate MCs), (2) Cytopore 2 (spherical CA microspheres), (3) Cultispher G (crosslinked gelatin MCs), (4) Cytodex 3, Cytodex 1 (spherical crosslinked dextran), and (5) QA52, DE53,

Table 3.7 Screening of hES cell cultivation on microcarrier biomaterials coated with VN and LN in MEF-CM after 5 days.²⁴ Adapted from ref. 24 with permission from Elsevier, Copyright 2014.

Microcarrier	Material	Cell attachment	Cell viability
Cytodex 1	Crosslinked dextran with <i>N,N</i> -diethylaminoethyl groups	Fair	Poor
Cytodex 3	Crosslinked dextran immobilized with denatured collagen on surface	Fair	Fair
Cytopore 1	Macroporous crosslinked dextran with <i>N,N</i> -diethylaminoethyl groups, charge density of 1.1 meq g ⁻¹	Poor	Poor
Cytopore 2	Macroporous crosslinked dextran with <i>N,N</i> -diethylaminoethyl groups, charge density of 1.8 meq g ⁻¹	Poor	Poor
CultiSphere-S	Crosslinked pharmaceutical grade porcine gelatin	Fair	Poor
Solo Hill H11-921	Crosslinked polystyrene modified with cationic trimethyl-ammonium	Poor	None
Solo Hill F102	Crosslinked polystyrene modified with cationic gelatin	Poor	None
Solo Hill C102	Crosslinked polystyrene modified with gelatin	Poor	None
Solo Hill G102	Crosslinked polystyrene modified with high silica glass	Poor	None
Solo Hill P102	Crosslinked polystyrene	Poor	None
Solo Hill PP102	Cationic crosslinked polystyrene	Poor	None
Solo Hill PF102	Crosslinked polystyrene modified with recombinant fibronectin	Poor	None

and DE52 (positively ionized, cylindrical CA MCs) and CM52 (negatively ionized surfaces).¹⁰¹ The macroporous gelatin MCs (Cultispher G) expressed low hES cell adhesion efficiency (23%). The hES cells had less cell adhesion (37–40%) on smaller (9–11 μm) MCs (Tosho 10 PR).¹⁰¹ This finding is probably because of the lack of nutrients for the hES cells in the macropores because of the restricted diffusive nutrients into the MCs. The hES cells on negatively ionized (CM52) MCs could not maintain pluripotency or cell proliferation. Only the hES cells on large spherical MCs (Cytopore 2, Culti-spher G, Cytodex 3, and Cytodex 1) grew, with 65–86% showing Tra-1-60 (pluripotent surface marker) at passage three when the MCs were not immobilized with Matrigels or ECMs in MEFs-CM.¹⁰¹ Therefore, the hES cells could be cultivated on MCs immobilized with Matrigels; the hES cells displayed long-term proliferation and supported their pluripotency in this research.

The cell adhesion efficiency had decreased on most MCs coated with Matrigels. For instance, the cell adhesion efficiency on positively ionized QA52 and DE53 MCs fell to 17–18% and 10–11%, respectively.¹⁰¹ The decreased cell adhesion was because of the Matrigel coating, which covered the positively ionized surface on the MCs. However, the Matrigel coating on the

MCs greatly increased the pluripotency and cell yield in long-term cultivation (3–12 passages); these cultivations expressed more than 90–92% viability, apart from the hES cells cultivated on MCs composed of gelatin (Cultispher G) and on negatively ionized MCs (CM52).¹⁰¹ Furthermore, positively ionized spherical MCs (Cytodex 1) and positively ionized cylindrical CA MCs (DE53) were chosen for the subsequent evaluation because of the high hPS cell expression of pluripotent markers when using the MCs in MEFs-CM. Compared to the Matrigel coatings, the LN-coated MCs (Cytodex 1 and DE53) could maintain long-term proliferation with a high pluripotent state of hES cells in MEFs-CM (Figure 3.18).¹⁰¹ The cell yield of hES cells on VN or hyaluronic acid was less than that on LN or FN and the cell yield of hES cells cultivated on FN-coated MCs was slightly less than that on LN.¹⁰¹ This observation indicates that a Matrigel or LN coating is important for the reliable long-term proliferation of hES cells (10–20 passages) on appropriate MCs (*e.g.*, Cytodex 1 and DE53) in MEFs-CM, although conventional MCs without a coating of ECMs or Matrigels do not maintain hES cell proliferation while keeping the hES cell pluripotency for more than 3–4 passages.

hPS cell cultivation on MCs typically requires a Matrigel coating and/or MEFs-CM. These treatments are xeno-containing cultivation factors and make it difficult to utilize hPS cells in stem cell therapy.

Heng and colleagues cultivated hES (H7 and HES-3) cells on crosslinked PS MCs (7602B, Thermo Fisher Scientific) coated with LN or VN in

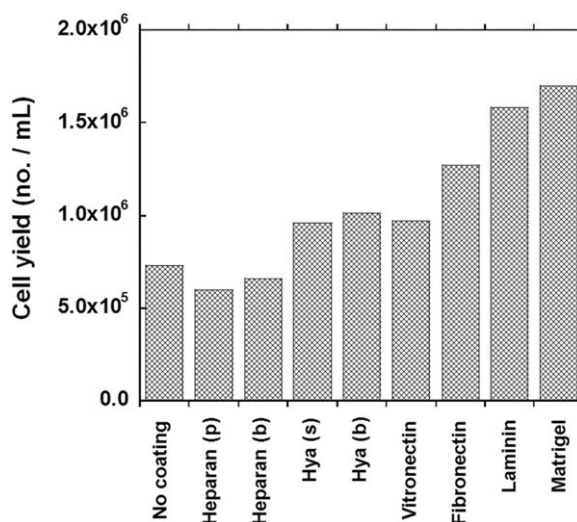


Figure 3.18 hES cell (HES-3) expansion on DE53 cellulose microcarriers immobilized with several ECMs in MEF-CM after two passages where Hya (b) is from bovine, Hya (s) indicates hyaluronic acid obtained from *Streptococcus*, heparan (b) is from bovine, and heparan sulfate (p) is obtained from heparan sulfate derived from pig. In total, hES cells at 1.60×10^5 cells mL⁻¹ were inoculated on 4 mg mL⁻¹ of microcarriers.¹⁰¹ Adapted from ref. 101 with permission from Elsevier, Copyright 2012.

chemically defined media (Table 3.6).⁵⁴ The attachment of LN or VN onto the MCs showed a coating surface concentration of 450 and 650 ng cm⁻², respectively, which were used to maintain hES cell proliferation.⁵⁴ The hES cells could proliferate long term on the MCs coated with LN or VN with a high expression (>90–92%) of TRA-1-60 and Oct-4 (pluripotent markers) for 20–22 passages by retaining typical karyotyping. The average fold enhancement in cell numbers on the MCs coated with both LN or VN per serial passage was 8.0–9.0.⁵⁴ The evaluation of EB and teratoma formation suggested that the hES cells maintained their ability to induce differentiation into the cells derived from all three germ layers. This is a novel study showing for the first time that hES cells could be proliferated on MCs for long passages (20–25 passages) under xeno-free and feeder layer-free cultivation conditions using defined media.

Although Heng's work⁵⁴ successfully demonstrated xeno-free and feeder layer-free cultivation of hES cells in chemically defined media, the investigators only evaluated a few ES (H7 and HES-3) cell lines. It is not clear whether the cell cultivation protocol and MCs used by Heng and colleagues are capable of using all hiPS and hES cell lines. It is necessary to synthesize or prepare MCs made of different types of polymeric materials and to establish optimal MCs for hPS cell cultivation in xeno-free and feeder layer-free cultivation conditions by referencing Heng's study.⁵⁴

Fan and his colleagues established a microcarrier system for hES cell culture without animal-derived components.⁶⁵ They found that PS microcarriers coated with vitronectin alone did not support hES cell cultivation in stirred suspension. However, microcarriers coated with vitronectin and human serum albumin (HSA), which were subsequently irradiated by UV light, led to enhanced seeding efficiency and retention of hES cells. hES cells expanded more than 20-fold per passage for multiple successive passages and without loss of cell pluripotency (Figure 3.19).⁶⁵ hES cells expanded on microcarriers were successively induced to differentiate into the cells derived from three germ layers, which indicate that their microcarrier system can be used for the self-renewal and specification of hES cells to therapeutically relevant cell types.⁶⁵ Such systems would be valuable for the envisaged use of hPSCs in drug discovery and translational medicine.

3.4.2 The 3D Cultivation of hPS Cells Embedded in Hydrogels (Microcapsules)

The microencapsulation of hPS cells is a valuable technique that prevents excessive aggregate agglomeration and protects against hydrodynamic shear while providing excellent diffusion of growth factors, gases, and nutrients *via* the microcapsule walls.¹⁴² Hydrophilic polymeric materials (hydrogels) are chosen to embed hPS cells in the hydrogels; these biomaterials include polyethylene glycol (PEG) derivatives, dextran, agarose, hyaluronic acid, and alginate.^{123,143,144}

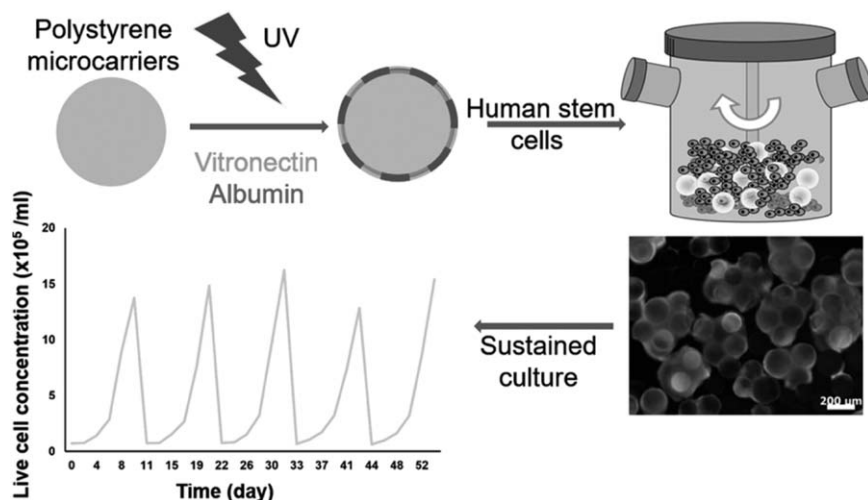


Figure 3.19 Development of microcarriers with recombinant vitronectin, human serum albumin, which were irradiated by UV light. hES cells could be expanded for five consecutive passages in xeno-free culture conditions.⁶⁵

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Alginate is one of the most important biomaterials frequently used for encapsulation. Alginate is a linear binary copolymer of 1,4-linked α -L-guluronic acid (G) and β -D-mannuronic acid (M) of greatly varying sequential structures and composition. Gelation of alginate occurs when multivalent cations, such as barium or calcium ions, ionically bind with blocks of guluronic residues among two different polymeric chains; this interaction generates 3D networks, leading to the formation of hydrogels.¹⁴⁵ Consequently, hPS cells embedded in alginate hydrogels are generated by dropping an aqueous alginate solution including hPS cells into a BaCl_2 or CaCl_2 solution. Alginates have some outstanding properties making these useful materials, such as good permeability, biosafety, and biocompatibility. Although alginates have bioinert properties and do not interact with hPS cells, several investigators have combined peptides with cell-binding sites or ECMs with alginate hydrogels using a grafting or blending technology. Several examples of cultivating hPS cells, which were microentrapped in hydrogels, are shown in Table 3.8.

Siti-Ismael and colleagues added hES (H1) cells to an alginate hydrogel that contained gelatin *via* the dropping addition of an aqueous alginate solution including gelatin and hES cells into a CaCl_2 solution using a needle.¹¹¹ The hES cells in the alginate hydrogel were cultivated over a period of 8–9 months. Cell aggregates formed within the alginate hydrogel, which maintained the pluripotent state and induced differentiation into the cells derived from three germ layers when they were cultivated in induction

Table 3.8 Study targeting proliferation of hPS cells microencapsulated in biomaterials (hydrogels).²⁴ Adapted from ref. 24 with permission from Elsevier, copyright 2014.

Cell line	Materials of microencapsulation	Shape	Medium	Fold expansion	Longest culture time	Ref. (year)
hESCs (H1)	Alginate-gelatin hydrogels	Spherical (2.3 mm diameter)	DMEM with KSR and bFGF	Not specified	260 days	111 (2008)
hESCs (SCED)	Alginate hydrogel	Spherical	MEF-CM	19.2 for 19 days	19 days	123 (2011)
hESC (H1, H9)	PEG-based hydrogel	Spherical	DMEM/F12 with KSR, bFGF	1.3–1.7-fold for 10 days	10 days	146 (2013)
hESCs (H1, H9, H13)	Crosslinked methacrylated hyaluronic acid	Discs with diameters of 3 mm and thicknesses of 2 mm	MEF-CM	4-fold for 4 days	20 days	112 (2007)

media.¹¹¹ These data indicate that hES cells can be kept in a pluripotent state in hydrogels without passage for a long time. Unfortunately, it was impossible in this study to ascertain the viability rate and expansion (fold increase) of the hES cells in the hydrogels. It was also not possible to evaluate whether the propagation of hES cells in the alginate hydrogels was arrested or decreased and whether the cells grew at a standard rate. However hydrogels, which include adhesion moieties such as ECM and are mixed into hydrogels, should lead to interesting matrices for hPS cell cultivation.

Serra and colleagues investigated various 3D cultivation systems for the entrapment of hES cells as (1) cells adhered on MCs in alginate hydrogels, (2) aggregated cells, and (3) single cells (Figure 3.16).¹²³ The microencapsulated hES cells were cultivated in a stirred tank bioreactor. Microentrapment promoted the cultivation of hES cell aggregates (30–110 μm) by maintaining the cell pluripotency, controlling the aggregate size, and avoiding the cells from hydrodynamic shear stress for 14 days; on the other hand, the cell viability reduced slowly from 94–96% to 4–6% after 1 week of culturing single hES cell embedded in the alginate microcapsule.¹²³ These results indicate that the microentrapment of single cells is not an optimal method for hES cell proliferation with supporting pluripotent state. An important improvement in metabolic activity and cell viability in aggregates of hES cells in the microcapsule of alginate was found compared with the metabolic activity and cell viability in the conventional 2D hES cell cultivation.

hES cells were adhered to Matrigel-coated MCs (Cytodex 3) and entrapped in spherical alginate hydrogels. The microentrapment of hES cell-attaching MCs in the alginate hydrogel greatly increased the cell viability and cell proliferation when compared with those in conventional 2D hES cell cultivation.¹²³ The combination of MC and microentrapment technology leads to excellent generation and storage of pluripotent hES cells. This strategy produces excellent cell proliferation, for example the 20-fold enhancement in cell density, when hES cells have sufficient surface area to propagate (Table 3.8).¹²³ This research indicates that the combination of cell microentrapment and MC engineering generates an appropriate system for the scalable generation of high-quality hPS cells maintaining their pluripotency, because the microentrapment offers a microenvironment having less shear stress and prevents the excessive coagulation of MCs and aggregates in hPS cell cultivation.

The alginate entrapment of hPS cells frequently shows chemical instability and weak mechanical properties such as weak resistance to osmotic swelling that sometimes leads to cell escape. However, Siti-Ismael and colleagues successfully achieved 8–9 months of cultivation of hPS cells entrapped in the alginate hydrogel.¹¹¹ The exterior of an alginate microcapsule is frequently coated with a polyelectrolyte complex to enhance the mechanical stability, where two different ionized polymeric materials are complexed together.¹⁴² The coating biomaterials are cationic polymers, such as PLL (poly-L-lysine), because the alginate polymers have anionic properties. PLL will bind to both the G and M blocks of the alginate *via* ionic interactions, but it binds more

strongly to alginates with higher M content.¹⁴² The positive charges of PLL on the outer surface of the microcapsule can be neutralized *via* doping of additional alginate to improve biocompatibility, making an alginate–PLL–alginate microcapsule. PEG, chitosan, and other cationic polymers could be used to replace PLL.¹⁴²

Jang and colleagues prepared a microcapsule of PEG-arm hydrogels using PEG3400 for hES (Novo, H9, and H1) cell cultivation in feeder layer-free conditions (Table 3.8).¹⁴⁶ The PEG-arm hydrogel was synthesized by the binding of vinylsulfone-functionalized 8-arm, 4-arm PEG, and 3-arm PEG to dicysteine-including oligopeptides with an intervening matrix metalloproteinase (MMP)-specific cleavage site (Ac-GCRD-GPQGIWGQ-DRCG-NH₂) *via* a Michael-type addition reaction (Figure 3.20).^{146,147} A higher expression of MMP-2 in H9 hES cells was generated than that using other MMP isomers. Then, the investigators chose the MMP-2-sensitive PEG-arm hydrogels for hES cell proliferation. The physical characteristics of the stem cell niche are key parameters for guiding cell fate and for supporting the differentiation or pluripotent state of stem cells.¹⁴⁸ There was a strong influence of PEG concentration (5–15%) and vinyl sulfone-containing PEG multi-arm number (8, 4, or 3) on hES cell shape in this research.¹⁴⁶ The PEG concentration and PEG arm numbers influenced the elasticity of the PEG-based hydrogels. hES cells generated apoptosis in the 3-arm PEG-based hydrogels, which were softer due to the shortage of hydrogel crosslinking compared to that in the 8-arm and 48-arm PEG-based hydrogels.¹⁴⁶ The relatively harder hydrogels from the 8-arm and 4-arm PEG-based hydrogels appear to be recommended for hES cell cultivation, which support cell pluripotency and viability.

Cell proliferation was shown to be highest with the 8-arm hydrogel with 10% PEG concentration, which expressed the highest amount of pluripotent genes (Sox2, Tert, Klf4, Nanog, and Oct3/4) on hES cells.¹⁴⁶ The 8-arm PEG-based hydrogel composed of 9–11% PEG was found to be preferable for cultivation in feeder-free 3D systems. The activity of alkaline phosphatase in hES cells in the 8-arm PEG-based hydrogel was almost the same as that in the 2D cultivation using conventional MEF feeder cells,¹⁴⁶ although an enhanced gene expression of pluripotency (Utf1, Sox2, Tert, Cdh1, and Klf-4) and increased expression of pluripotent proteins (Tra-1-81, Tra-1-60, Nanog, Oct3/4, and SSEA-4) were observed in the 3D-cultivated hES cells compared to those in the hES cells cultivated on MEFs (2D cultivation).¹⁴⁶ This evidence shows that the chemically defined, acellular niches generated using PEG-arm hydrogels have the ability to maintain hES cell proliferation.

3.5 hPS Cell Cultivation on PDL-coated Dishes with Small Molecules

Rho signaling is necessary for retaining cell–cell contacts in hES cells.⁸¹ The use of a specific ROCK inhibitor indicates that cell–cell attachment is reversibly regulated and is not dispensable for hES cell proliferation.

Meng and colleagues⁴⁷ and Harb and colleagues⁸¹ described how it was possible for hES cells to be cultivated on PDL-coated dishes in media containing Y27632 (ROCK inhibitor). The hES cells support their ability to self-renew, their pluripotency, and their ability to induce differentiation into the cells derived from three germ layers. In conventional cultivation, Y27632-including media is typically changed for fresh media without ROCK inhibitor 1 day after seeding or thawing dissociated cells because ROCK inhibitor is able to facilitate hPS cell viability and adhesion but not proliferation.⁴⁷ However, Y27632 always exists in the cultivation media in this study⁴⁷ because the hES cells were easy to remove from the culturing surface if refeeding occurred without ROCK inhibitor in the cultivation media.

Long-term cultivation (>10–30 passages) of hES cells on PDL-coated plates in defined media with Y27632 supplement must be investigated to analyze whether hPS cells are able to expand and support their pluripotent state and ability to induce differentiation into the cells derived from three germ layers with these systems.

3.6 Conclusion and Future Perspectives

Some xeno-free and feeder-free cultivation methods have been recently developed; hPS cells are able to be cultivated on (1) PDL-coated plates in TeSR2 supplemented with Y27632, (2) StemAdhere-coated plates in TeSR2 media, (3) CELLstart (albumin and FN)-coated plates in StemPro, TeSR2, or another hPS cell cultivation medium; (4) Synthemax (a peptide derived from VN) plates in StemPro, TeSR2 media, or Essential 8 media; (5) VN-coated plates in TeSR2 media or Essential 8 media; or (6) LN-511 or LN-521-coated plates in TeSR2 media or Essential 8 media. However, not all hPS cells are able to expand and support their pluripotent state in each of the above serum-free cultivation conditions. Future establishment of coating biomaterials and cultivation matrices, including microfibers, nanofibers, MCs, hydrogels, and plates, is important for the long-term cultivation of hPS cells and for the establishment of appropriate hPS cell cultivation media.

The present MC biomaterials used for hPS cell cultivation are restricted to crosslinked gelatin, dextran, and PS beads. This is because the typical MCs used for hPS cell cultivation are commercially available beads (Tables 3.6 and 3.7). It is important to prepare MCs having a polymeric (and surface) design that holds hPS cell proliferation and supports hPS cell pluripotency for a long-term 2D cultivation. MCs that are immobilized with specific polymeric materials (AETMA-Cl/DEAEA, APMAAm, PMVE-*alt*-MA, and PMEDSAH,) or immobilized with optimal peptides (Oligo-HBP1 and VN-PAS) are considered to support many types of hPS cells by retaining their pluripotent state in a defined medium to long term (>15–30 passages).

At present, no *meta*-analysis has been done to evaluate why specific polymeric materials with a certain chemical scheme maintain some hPS cell lines. Several specific polymeric materials have been reported to maintain the pluripotency of hPS cells, but it is still important to re-evaluate these

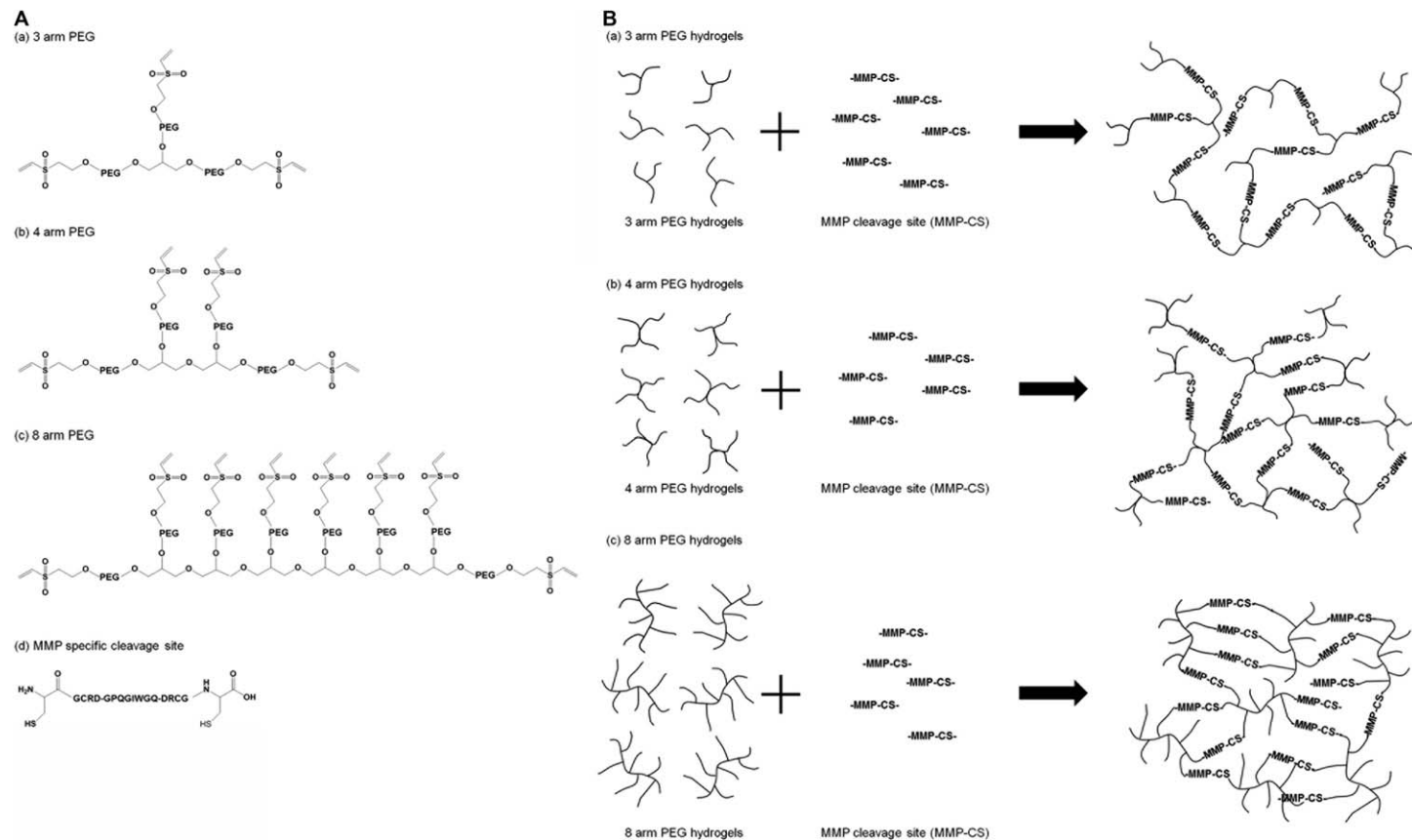


Figure 3.20 Chemical scheme of PEG-arm hydrogel. (A) Chemical scheme of a 3-arm PEG (a), 4-arm PEG (b), 8-arm PEG (c), and a peptide with MMP-specific cleavage sites (d). (B) Chemical reaction of 3-arm PEG hydrogel (a), 4-arm PEG hydrogel (b), and 8-arm PEG hydrogel (c).²⁴
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using various cell lines and cultivation media, and the data need to be verified by other investigators. The synthetic biomaterials seem to need the production of ECMs from hPS cells or the sorption of proteins from a conditioned media of feeder cells (or another cultivation medium) because of the heparin-like characteristics of the biomaterials having a growth factor-adsorbed surface. A biological evaluation of synthetic biomaterials and surface coatings that maintain hPS cells would contribute to developing the most efficient synthetic material surface architecture or coating biomaterials for hPS cell cultivation.

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CHAPTER 4

Differentiation Fates of Human ES and iPS Cells Guided by Physical Cues of Biomaterials

4.1 Introduction

It is evident that people frequently experience the loss or damage of tissues or organs as a result of birth defects, accidents or disease.^{1,2} Regenerative medicine and tissue engineering may be greatly aided by the use of stem cells, such as hES cells³ and hiPS cells.^{4–6} Not only do stem cells themselves regulate their characteristics, including appropriate differentiation and the maintenance of pluripotent state, but so too do their microenvironments. As a result, the use of synthetic and natural polymer materials to mimic stem cell microenvironments and niches can help to generate significant numbers of stem cells and help to produce the widely differentiated cells that are necessary for *in vitro* translational medicine. Hormones, extracellular matrix (ECM) conditions and small chemical molecules are among a number of biological cues that determine the pluripotency of stem cells and their differentiation fates (Figure 4.1).^{7–12} However, researchers have only of late started to consider how external forces (*e.g.*, light signals, magnetic forces, and electrical forces),^{13,14} mechanical forces (*e.g.*, shear stress imposed by cultivation medium and cyclic stretching of biomaterials),¹⁵ polymeric biomaterial stiffness,¹⁶ cell shape, and other such physical cues impact on the induction of stem cells (Figure 4.1). Currently, no review studies have examined in detail the matter of the physical cues where cell cultivation polymer materials provide for the differentiation of hiPS and hES cells. There have, however, been a number of outstanding

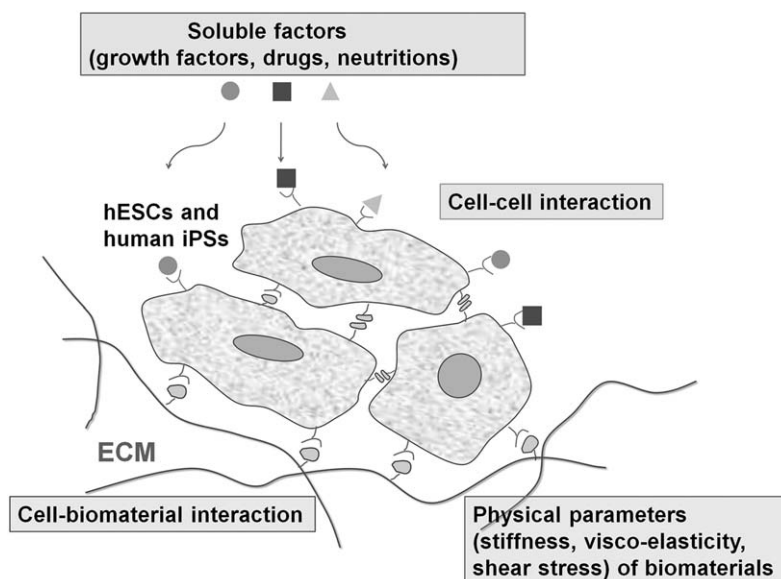
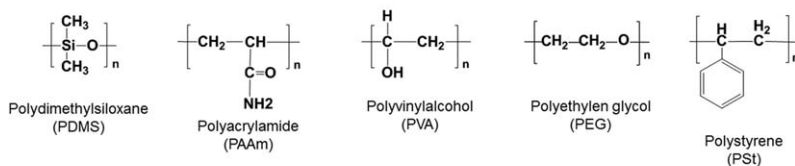


Figure 4.1 Schematic illustration of the niche and microenvironment of stem cells and their control by the following factors: (a) soluble factors, such as bioactive molecules, nutrients, and cytokines or growth factors; (b) cell–cell interaction; (c) material–cell interaction. Physical characteristics of materials (d) also control stem cell fate.² Adapted from ref. 2 with permission from American Chemical Society, Copyright 2011.

review and original works on the differentiation of mouse ES cells and adult stem cells on these materials.^{7,9,10,16,17} The protocols and differentiation methods employed with hiPS and hES cells are significantly different from those employed with adult stem cells because of the much higher pluripotent state of hPS cells (hiPS and hES cells). Furthermore, hiPS and hES cells cannot generally be cultivated in conventional polystyrene cultivation plates,^{1,2} because these induce random (spontaneous) differentiation of hiPS and hES cells. Likewise, adult stem cells can expand and differentiate in polystyrene plates and they can be controlled by the induction medium.

Therefore, this chapter discusses the physical cues of synthetic and natural polymeric materials that lead to the differentiation of hES cells and hiPS cells into several different lineages. Such lineages include dopamine-secreting neurons, neural cells, insulin-secreting beta cells, hepatocytes, retinal pigment epitheliums, osteoblasts, chondrocytes, and cardiomyocytes. In this chapter, the physical cues of materials that we focus upon are (1) the elasticity of polymeric biomaterials, (2) the topography of polymeric biomaterials, and (3) the mechanical forces associated with materials (electrical stimulation *via* materials and stretching of materials) used for hPS cell cultivation.

(a) Synthetic polymer



(b) Biopolymer

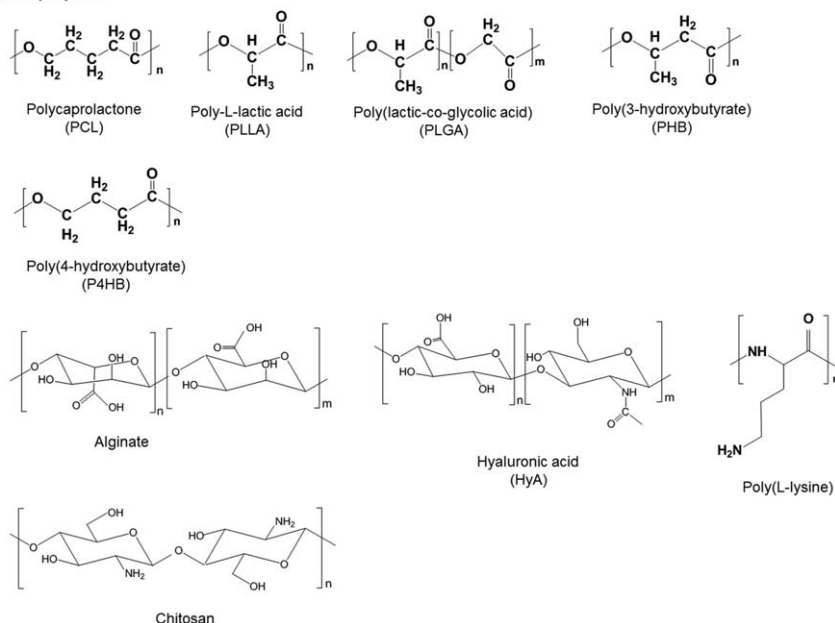


Figure 4.2 Chemical schemes of synthetic polymers (polystyrene [PSt], polyethylene glycol [PEG], polyvinyl alcohol [PVA], polyacrylamide [PAAm], and polydimethylsiloxane [PDMS]) (a) and biomacromolecules (chitosan [CS], alginate, poly-L-lysine [PLL], hyaluronic acid [HyA], poly(4-hydroxybutyrate) [P4HB], poly(3-hydroxybutyrate) [PHB], poly(lactic-co-glycolic acid) [PLGA], poly-L-lactic acid [PLLA], and poly-ε-caprolactone [PCL]) (b) utilized as scaffolds, hydrogels, and substrates for the expansion and induction of stem cells.¹⁷ Reproduced from ref. 17 with permission from American Chemical Society, Copyright 2013.

These are discussed in the following sections. Figure 4.2 contains a summary of the chemical schemes of the materials discussed in this chapter. Table 4.1 provides a summary of a number of the proteins and genes used in this chapter, which are commonly used to evaluate stem cell differentiation into the cells derived from some lineages and their descriptions. Some methods of staining cells used to evaluate the cells derived from some lineages are shown in Table 4.2.

Table 4.1 Proteins and genes to examine the induction of stem cells into specific lineages.¹⁷ Adapted from ref. 17 with permission from American Chemical Society, Copyright 2013.

Differentiation lineage	Gene or protein	Specification
Osteoblasts	Runx2 (CBF α 1)	Early osteoblast marker
	Osterix	Early osteoblast marker
	Osteocalcin (OCN)	Late osteoblast marker
	Osteopontin (OPN, Spp1)	Late osteoblast marker
	Alkaline phosphatase (ALP)	Early osteoblast marker
	Bone sialoprotein (BSP)	Osteoblast marker
	Collagen type I (Col I)	Osteoblast marker
Chondrocytes	Sox 9	Chondrocyte marker
	Col2A1	Chondrocyte marker
	Aggrecan (ACAN)	Chondrocyte marker
	Collagen type II (Col II)	Chondrocyte marker
	Collagen type X (Col X)	Chondrocyte marker
	Cartilage oligomeric protein (COMP)	Chondrocyte marker
Adipocytes	Adipocyte lipid-binding protein (ALBP)	Adipocyte marker
	PPAR γ	Adipocyte marker
	aP-2	Adipocyte marker
	Lipoprotein lipase (LPL)	Adipocyte marker
Cardiomyocytes	Cardiac troponin T (cTnT)	Cardiomyocyte marker
	Desmin	Cardiomyocyte marker
	Myosin heavy chain (MHC)	Cardiomyocyte marker
	Myosin light chain (MLC)	Cardiomyocyte marker
	Nkx2.5	Cardiomyocyte marker
	GATA-4	Cardiomyocyte marker
Muscle	Smooth muscle α -actin (SMA)	Smooth muscle cell marker
	α -actin	Smooth muscle cell marker
	Calponin 1	Smooth muscle (contractile) marker
	Myocardin	Smooth muscle cell marker
	Smoothelin	Smooth muscle cell marker
	Collagen type 4	Myogenic marker
	Desmin	Myogenic marker
	Pax3	Myogenic marker
	Pax7	Myogenic marker
	Myogenin (MYOG)	Myogenic marker
	MyoD, MyoD1	Myogenic marker
Neural cells	Nestin	Neural stem/progenitor cell marker
	ENO2	Neural cell
	β -tubulin III or β -III tubulin (Tuj-1)	Neuronal marker
	Tyrosine hydroxylase (TH)	Neuronal marker (dopamine secreting cells)
	Neurofilament light chain (NEFL, NFL)	Neuronal marker
	Neurofilament heavy chain (NFH)	Neuronal marker
	Microtubule-associated protein 2 (MAP2)	Mature neuronal marker

Table 4.1 (Continued)

Differentiation lineage	Gene or protein	Specification
	Glial fibrillary acidic protein (GFAP)	Astrocyte marker
	Galactosylceramidase (GalC)	Oligodendrocyte marker
	RIP	Mature oligodendrocyte
	O4	Oligodendrocyte marker
	CNPase	Oligodendrocyte marker
Endothelial cells	Flk-1	Endothelial cell marker
Hepatocytes	α -fetoprotein (AFP)	Early hepatocytes
	Albumin (ALB)	Mature hepatocytes
	Epithelial cell adhesion molecule (EpCAM)	Hepatic stem cells, hepatoblasts
	Neural cell adhesion molecule (NCAM)	Hepatic stem cells (and neural cells)
	E-cadherin (CDH1)	Hepatic stem cells (and ESCs, iPSCs)
ESCs, iPSCs	Oct3/4	Pluripotent marker
	Sox2	Pluripotent marker
	Nanog	Pluripotent marker

Table 4.2 Methods of staining to examine the induction of stem cells into specific lineages.¹⁷ Adapted from ref. 17 with permission from American Chemical Society, Copyright 2013.

Staining method	Detection site	Characterization
Paxillin labeling	Paxillin	Focal adhesion
Phalloidin F-actin	F-actin	Focal adhesion
Vinculin	Focal adhesion protein	Focal adhesion
Oil red O	Oil droplet	Adipocytes
Nile red	Oil droplet	Adipocytes
Alizarin red	Calcium	Osteoblasts
von Kossa	Calcium phosphate	Osteoblasts
Alkali phosphatase	Alkali phosphatase activity	Osteoblasts
Safranin-O	Proteoglycan	Chondrocytes
Alcian blue	Proteoglycan	Chondrocytes
Toluidine blue	Proteoglycan	Chondrocytes
DAPI	DNA	Nucleus
Hoechst	DNA	Nucleus
Masson's trichrome	Tissue	Connective tissue, nuclei, cytoplasm
Haematoxylin and eosin (H&E)	Tissue	Connective tissue, nuclei, cytoplasm

4.2 Induction Protocols of Human Pluripotent Stem Cells

We consider some induction protocols of differentiation to be used for hPS cells. Following on from this, we outline the physical effects of materials on

hPS cell differentiation. We propose a number of methods for differentiating hPS cells into specific cell lineages (Figure 4.3).¹⁸ The induction protocols of adult stem cells are, of course, relatively easy. These include cultivation of stem cells on scaffolds in induction media or in cell culture dishes. However, while human adult stem cells can expand and retain their stem cell state in traditional tissue culture polystyrene (TCP) plates, hPS cells should be cultivated in colonies either on Matrigel, on specific feeder layers, or on materials in order for feeder-free cultivation to keep their pluripotent state. Mouse PS cells (mPS cells), such as mouse iPS and mouse ES cells, are cultivated more easily than hPS cells. mPS cells typically expand in gelatin-coated plates in cultivation medium including LIF (leukemia inhibitory factor), whereas hPS cells cannot maintain their pluripotent state in gelatin-coated plates. Moreover, whereas the differentiation ability of human adult stem cells is extremely restricted (*e.g.*, cells derived from the mesoderm lineage), in most cases hPS cells have the potential to induce the differentiation into many tissue types of the cells derived from all three germ layers.

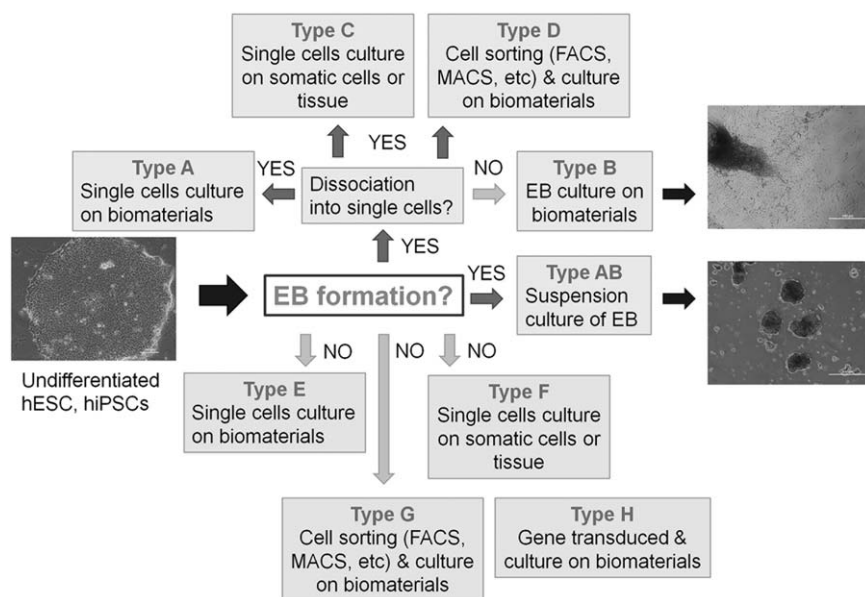


Figure 4.3 Induction protocols of hPS cells into optimal cell lineages. Induction of hPS cells through EB generation (Types AB, A, B, C, and D) and without EB generation (Types E, F, G, and H). Single or aggregate cells are inoculated onto materials in the Type A and E protocols. Cells are cultivated on somatic cells for induction in the Type C and F protocols. Cell sorting using FACS, MACS, or cell selection based on cell morphologies is used in the Type D and G protocols. Cell selection is done by either gene transfection or gene transduction in the Type H protocol.¹⁸ Reproduced from ref. 18 with permission from the Royal Society of Chemistry.

A typical (easy) method for the differentiation of hPS cells involves the generation of an embryoid body (EB) for differentiation (Type AB and A–D in Figure 4.3). EB generation is an easy way to obtain cells, which are induced into differentiation toward all three germ layer lineages as the pluripotent state of hPS cells cannot be retained during EB generation in differentiation media, whereas hPS cells start spontaneous differentiation in EB generation. It has been demonstrated that multicellular interactions within the three-dimensional (3D) hPS cell aggregates termed EBs mimic aspects of embryonic differentiation processes *in vitro*.¹⁹ In particular, the differentiation of hPS cells into endoderm cells, such as hepatocytes and β cells, or into neural lineage cells, which are relatively difficult to prepare from hPS cell differentiation, has been made possible *via* the process of EB generation. However, recent methods have tended not to include this approach for the purposes of enhancing the purity of targeted induced cells.^{20–23} This is principally due to the fact that EBs include several different lineages of cells, with the result that it is necessary to carry out the purification process of the desired cell lineage by means of several sorting methods.

4.2.1 EB Formation

When hPS cells are cultivated in non-adhesive cell cultivation plates in suspension medium without bFGF (FGF-2), PS cells change structures, spheroid in nature, which are referred to as EB (suspension culture, see Figure 4.4). While significant numbers of EB can be formed with ease in suspension cultivation, EBs in such cultivation conditions generally exhibit many different sizes (Figure 4.4A).²⁴

EBs are also formed by a hanging drop protocol (Figure 4.4B and C), where a single drop (typically 10–50 μ l) of cultivation medium is formed from the lid of a Petri dish or from a lid pierced with tiny holes, and PS cells are cultivated in these drops. The hanging drop protocol can generate fairly uniformly sized EBs.²⁵ However, it is not easy to generate large numbers of EBs at once. It appears that the geometry of 3D aggregates (*i.e.*, surface area to volume ratio and size) is an important factor in influencing differentiation into reliable cell lineages (Figure 4.4).^{26,27}

Dahlmann *et al.* described a method for the generation of uniform EBs which makes use of a micropatterned polymeric mold²⁵ and has been proposed by some other investigators.²⁷ They made a microwell plate with agarose using soft lithography technology to make uniformly sized EBs akin to those produced with the hanging drop technology (Figure 4.5).²⁵ Initially, hydrophilic-like silicone was cast either (1) on a bicycle rear retroreflector, which had about 800 corner cube apertures of 95 mm diameter or (2) on commercial AggreWell™ 800 plates (each well 15.6 mm in diameter) that contained structured PDMS surfaces comprising arrays of 300 inverse pyramidal microwells with side lengths of 800 μ m for the generation of silicon master plates (Figure 4.5A).²⁵ After a 20 minute curing period, the solidified silicon master was peeled from the retroreflector or AggreWell™ 800 plate.

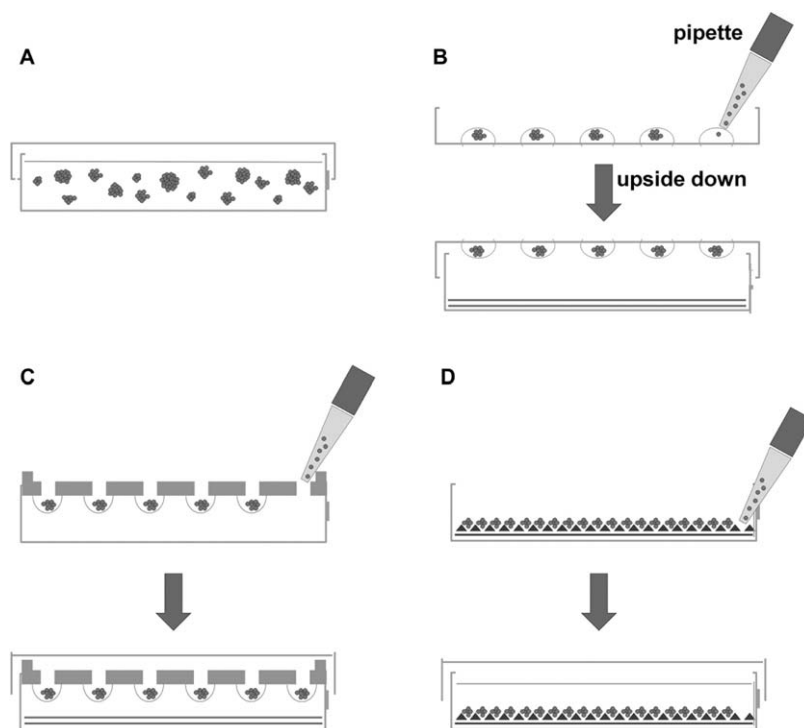


Figure 4.4 Schematic illustration of EB generation. (A) EB generation in suspension cultivation. hPS cells were cultivated in non-adherent cell plates. (B) EB generation by a hanging drop method. hPS cell solution was dropped onto the lid of a plate, which was then kept upside down. (C) EB generation by a hanging drop method. hPS cell solution was dropped onto a lid with a hole in it, creating a hanging drop of hPS cell solution. (D) EB generation on a micropatterned biomaterial.¹⁸ Reproduced from ref. 18 with permission from the Royal Society of Chemistry.

The silicon master plate was set on top of liquid agarose–DMEM media in dishes at 65 °C, while the silicon masters were allowed to swim up (Figure 4.5B). After agarose solidification, the silicon master was taken out, and the agarose micropatterned plate was subsequently generated. hES cells were inoculated onto each corner cubic-patterned or inverted pyramidal-patterned surface in the micropatterned agarose plate in order to generate uniformly sized EBs (Figure 4.5C), such as those generated in the hanging drop protocol.²⁵ EB size was found to be regulated by inoculating cell density in each microwell; 2666, 2000, 1333, and 666 cells were inoculated in each microwell, and hES cells were differentiated into cardiomyocytes (Figure 4.6).²⁵ This research showed that efficient cardiomyogenic differentiation of hES cells was observed with the generation of extensively homogeneous sized EBs when using specifically sized agarose microwells and cultivating the EBs in cardiomyocyte induction medium.

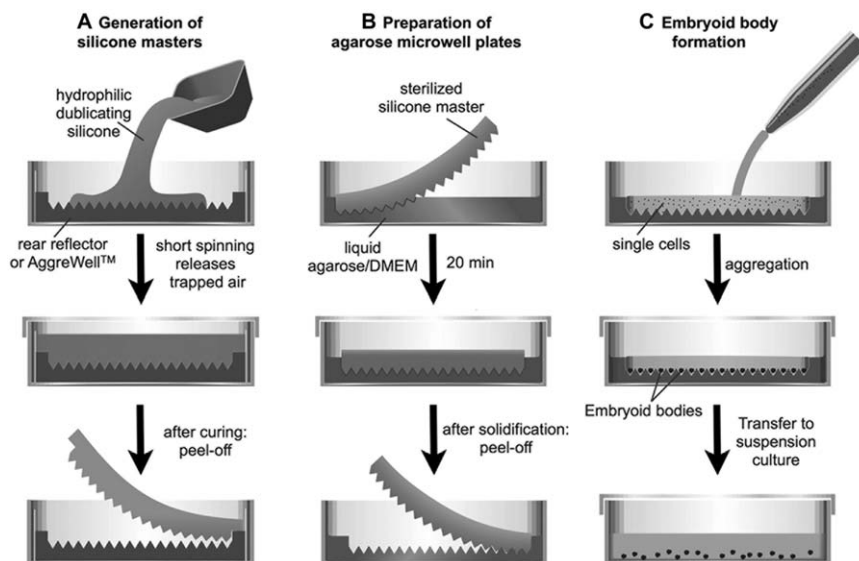


Figure 4.5 Schematic picture of the multistep processes used to make agarose microwells for EB generation of hPS cells. (A) Hydrophilic-like silicone is cast either onto a commercially available AggreWell plate or onto the microstructured materials of a bicycle rear retroreflector. The silicone master is peeled off after curing. (B) A sterilized reusable silicone master is placed in liquid agarose/DMEM (1.5% w/v) and generated to swim-up. A mirror-inverted patterned agarose–DMEM interface is generated by solidification. (C) hPS cells are inoculated into the agarose–DMEM microwells. Cells settle by gravitation and self-aggregate to generate EBs, which can be recovered for further culture.²⁵ Reproduced from ref. 25 with permission from Elsevier, Copyright 2013.

The use of micropatterned plates for the generation of EBs supports the advantage of forced aggregation, such as in the hanging drop method (high reproducible size of EBs). It also provides the advantage of suspension-based EB generation, which is an effortless, scalable, simple and rapid protocol.

4.2.2 Induction of hPS Cells by EB Generation

The purification, culture, and/or differentiation of cells into appropriate lineages should be processed after EB generation from hPS cells. EB generation can be used to start the induction of hPS cell differentiation. It should be possible to categorize the several methods of hPS cell induction into appropriate cell lineages, which use EB generation into Types A, B, C, D, and AB, as described in Figure 4.3. We discuss some methods of hPS cell differentiation by EB formation, including Types A–D and AB in this section.

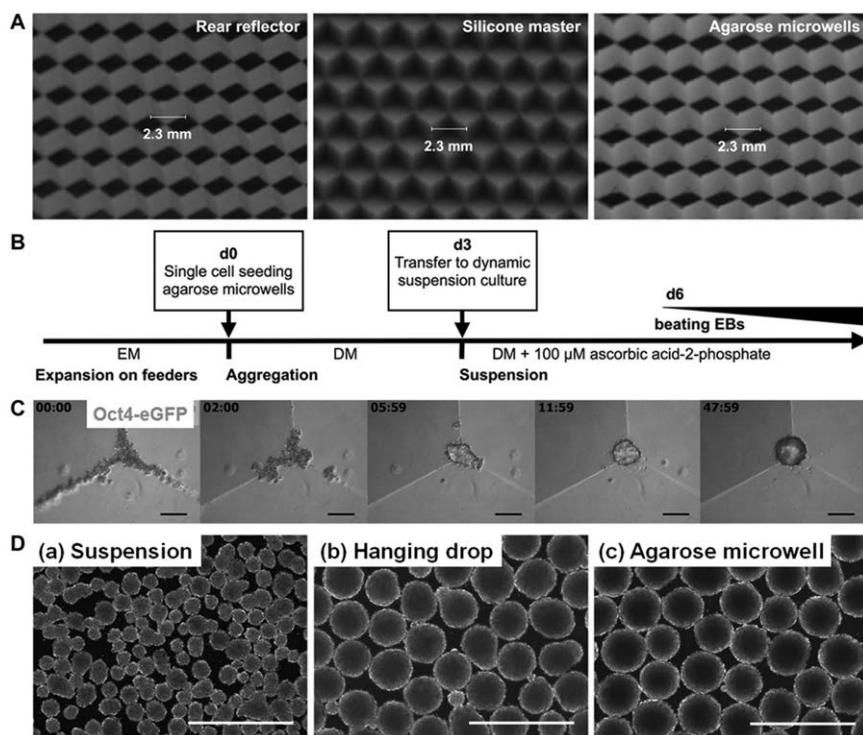


Figure 4.6 EB generation and cardiomyogenic induction of hPS cells in agarose microwell plates (AML). (A) Corner cube surface topographies of a silicone master and a rear retroreflector and the AML plates. (B) Method for serum-based hPS cell induction generated on AML plates. DM, differentiation medium; EM, expansion medium. (C) Time course of EB generation. Merge of brightfield and Oct4-controlled eGFP fluorescence (green). EB generation was complete within 12 h. Time scale = HH:mm, scale bars = 1 mm. (D) Morphological appearance of EBs formed in suspension cultivation (a), in hanging drops (b), and on AML (c) on day 3.²⁵ Reproduced from ref. 25 with permission from Elsevier, Copyright 2013.

4.2.2.1 Type AB Differentiation of hPS Cells

In Type AB differentiation, the EB is prepared from pluripotent hPS cells. It is then cultivated in suspension in several kinds of differentiation medium, including antagonists and/or agonists of signal pathways.^{25,28,29} The differentiation from hES cells into cardiomyocytes that was reported by Dahlmann and his colleagues²⁵ is regarded to be classified as Type AB induction.

Dahlmann and his colleagues prepared several different-sized EBs by controlling the inoculating densities of hES cells on micropatterned agarose plates and cultivated EB in cardiac differentiation medium as described in Section 4.2.1.²⁵ A beating EB was first detected on days 5–7 and consistently enhanced until days 9–11. A population of approximately 100% beating EBs

was obtained under the conditions that used inoculating densities of 666–2000 cells per microwell.²⁵ A flow cytometry assay of dissociated EBs on day 10 showed that the highest yield of cardiomyocytes was generated from the conditions that used an inoculating density of 666 cells per microwell. Higher cell numbers per microwell generated fewer cardiomyocytes. However, when the purity of the cardiomyocytes was evaluated, 1333 cells per microwell generated the highest ratio of cardiomyocytes having cTnT⁺ (cardiac troponin T), which was up to 65%.²⁵ This study confirmed the benefits of a uniform size of EB generation to obtain efficient induction of cardiomyocytes from hES cells in cardiomyocyte differentiation medium.

4.2.2.2 Type A Differentiation of hPS Cells

In the Type A differentiation method, the EB is formed from hPS cells, and cells in the EB are dissociated into small aggregates or single cells. Then, the cells are cultivated on some two-dimensional (2D) biomaterials and on 3D biomaterials such as microcarriers, hydrogels, or scaffolds to proceed further induction.^{15,30–39}

Chondrogenic induction of hiPS (SC802A-1) cells using EB generation *in vitro* was studied by Ko and his colleagues.³⁰ The EB was made into single cells after cultivation of the EB for 10 days. The cells were induced into chondrogenic differentiation using pellet cultivation or in cell cultivation entrapped in an alginate hydrogel (Figure 4.7).³⁰ Oct3/4, SSEA-4, and Nanog (pluripotent markers) disappeared in the immunochemical evaluation of hiPS cells after chondrogenic differentiation of hiPS cells, however BMP-4 (mesodermal gene marker) was newly expressed. Glycosaminoglycan content increased compared to EB and hiPS cells after 21 days of differentiation. Chondrogenic properties, such as abundant matrix formation and lacuna generation of proteoglycans as analyzed by Safranin O staining, were found to be higher in hiPS cell-derived chondrocytes than in hBMS (human bone marrow stem) cell-derived chondrocytes.³⁰ The chondrogenic markers (aggrecan, collagen type II, and SOX-9) expressed in hiPS cell-derived chondrocytes was observed to be similar or higher than hBMS cell-derived chondrocytes. Very low levels of osteogenic (collagen type I and Runx2) and hypertrophic (collagen type X) markers were observed in hiPS cell-derived chondrocytes compared to hBMS cell-derived chondrocytes.³⁰ The epigenetic assays and above data indicated that hiPS cell-derived chondrocytes expressed fewer hypertrophic properties and more chondrocyte properties than hBMS cell-derived chondrocytes.

The hiPS cell-derived chondrocytes in alginate hydrogel and in pellet was transplanted into defects of osteochondrocytes generated on patellar grooves in immune-suppressed rats and analyzed after 12 weeks (Figure 4.8A). The defects where hiPS cell-derived chondrocytes displayed extensive cartilage repair compared to control groups, and most of the cells in the regenerated cartilage composed of the cells, which were transplanted, as displayed in Figure 4.8B.³⁰

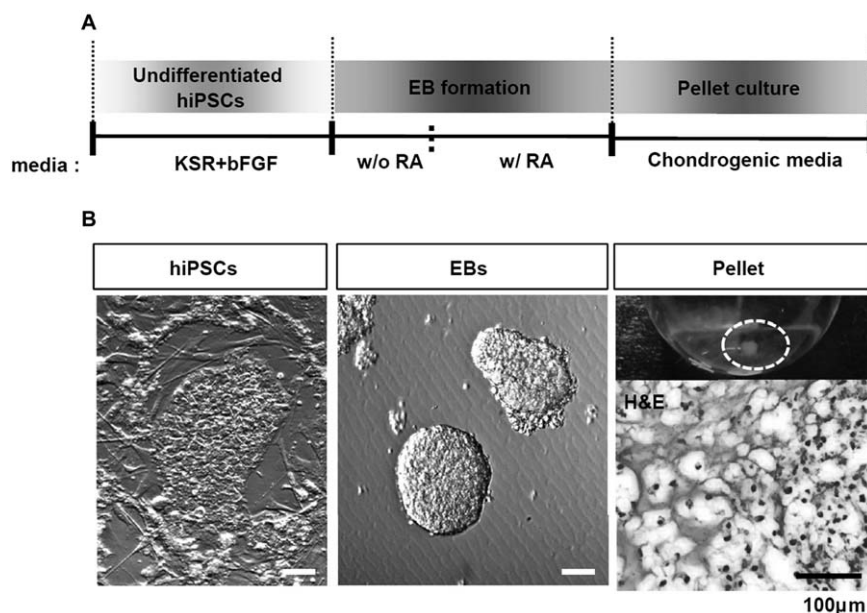


Figure 4.7 Formation of chondrogenic pellets. (A) Timeline protocols for chondrocyte induction of hiPS cells. (B) Undifferentiated hiPS cells and EBs in a cultivation plate and hiPS cell-derived chondrogenic pellets in a cultivation tube with H&E staining.³⁰

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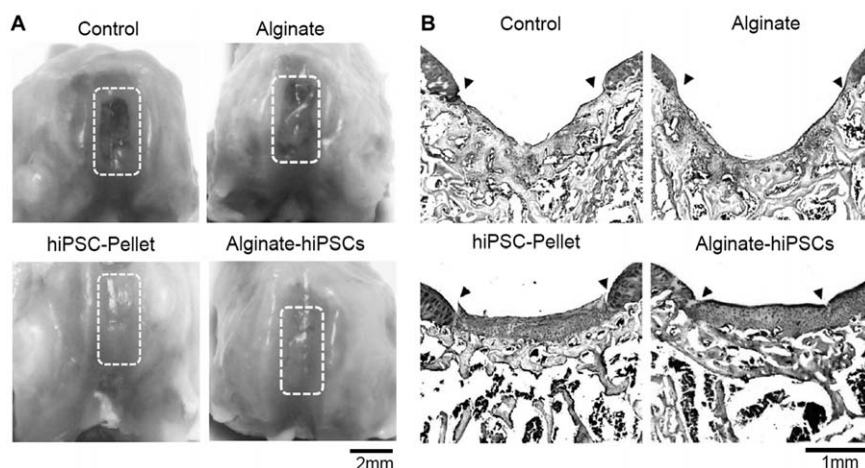


Figure 4.8 *In vivo* repair of osteochondral defects using hiPS cell-derived chondrocytes. (A) Gross pictures of defects, which were treated with chondrocytes induced from hiPS cells, alginate hydrogels, and chondrocytes induced from hiPS cells entrapped in alginate or without treatment (control). Dotted outline indicates the defect margin. (B) Histological pictures of a defect based on Safranin O staining. The arrows indicate the margin of the defect.³⁰

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4.2.2.3 Type B Differentiation of hPS Cells

In the Type B differentiation method, EBs formed from hPS cells are subsequently cultivated on microcarriers (3D), in hydrogels (3D), on scaffolds (3D), or on biomaterials (2D), all of which make the cells in the EB have cell-material interactions (Figure 4.1).^{27,28,37,40–57}

Various sizes of EB from hES cells were prepared using different sizes of microwells by Mohr and his colleagues.²⁷ Then, the EB was re-inoculated and cultivated in EB medium in gelatin-coated plates. They found that the highest percentage of contracting EB could be generated using an intermediate size (300 μm) of microwell, suggesting hES cell differentiation into cardiomyocytes, although there was a similar percentage of cells (3%) expressing the cardiac marker MLC2a (myosin light chain 2A) in EBs prepared in 300 μm microwells compared to EBs formed in 100–300 μm microwells. The results indicate that smaller EB is difficult to generate contractions. However, contracting EB having smaller size is easily enriched in cardiomyocyte compared to EB having larger size where cardiomyocyte makes up proportionately smaller fractions. This is explained as a beating EB can be formed even if only trace amounts of cardiomyocyte exist within the EB, suggesting that the bigger EB has a higher probability of containing cardiomyocytes than the smaller EB.

The differentiation potential of EBs generated from hES cells into keratinocyte precursor cells was evaluated by Ji and his colleagues when the EB was cultivated in plates with different coatings, including Matrigel, fetal bovine serum (FBS), collagen type I, and gelatin.⁵⁷ Cells that were cultivated in plates immobilized with gelatin showed the highest expression of K14 (keratin 14) marker in comparison to those cultivated in other plates, suggesting that hES cells cultivated in gelatin-coated plates could strongly induce differentiation into keratinocyte precursors.⁵⁷

4.2.2.4 Type C Differentiation of hPS Cells

In the Type C differentiation method, EBs formed from hPS cells are cultivated on some tissues or somatic cells, which induce the differentiation of hPS cells into desired types of cells.⁵⁸ This protocol of hPS cell induction is not as frequently used as the Type D, B, and A induction protocols through EB generation.

4.2.2.5 hPS Cell Induction in Type D

In the Type D differentiation protocol, single cells are prepared from EBs generated by hPS cells, and some types of desired cells are chosen based on their morphology (for example, rosette structure) or are sorted by FACS (fluorescence-activated cell sorting) or MACS (magnetic-activated cell sorting).^{47,49,51,53,59–65} This originates from the fact that the EB contains many types of cells. Then, the desired cells are purified using the cell sorting method.

Wang and his colleagues obtained NCSCs (neural crest stem cells) from hES and hiPS cells using EB generation.⁶⁴ Then, the EB was inoculated and cultivated on CELLstart-coated plates (mixture of albumin and fibronectin) in neural differentiation medium for 7 days. Subsequently, colonies exhibiting rosette morphologies were mechanically chosen and cultivated in suspension in serum-free medium for 7 days.⁶⁴ The cells were dissociated into single cells and cultivated on CELLstart-coated dishes for another 3 days. Subsequently, the cells were cultivated in suspension in serum-free medium for 7 days to generate spherical cell aggregates. The cell aggregates were reseeded in CELLstart-coated dishes in which the cells then migrated out from the attached spherical cell aggregates and grew to confluence (Figure 4.9A).⁶⁴ Furthermore, NCSCs continued to be cultivated in a monolayer (Figure 4.9A). The cells induced from different hiPS and hES cells showed 78–96% of native levels of NCSC marker, including HNK1, p75, nestin, and AP2, which depends on the cell lines (Figure 4.9B). This result suggests that this protocol can form highly pure and homogeneous NCSCs derived from hPS cells. However, this method needs different cultivation protocols (suspension cultivation and monolayer cultivation) and mechanical selection of the cells (for example, cell selection with rosette morphology).

Dubois and his colleagues used dissociated cells from EB, which were cultivated in some types of induction medium to prepare cardiomyocytes from hES cells (HES2).⁵⁹ The percentage of cardiomyocytes in the dissociated cells was enhanced by FACS using the cardiomyogenic marker of signal-regulatory protein alpha, SIRPA (CD172a).⁵⁹ The cardiomyogenic specificity of the sorted cells was investigated by flow cytometry evaluation for cTnT (cardiomyogenic troponin T).⁵⁹ Co-staining of cTnT and SIRPA from flow cytometry indicated apparent co-expression of both markers, suggesting that

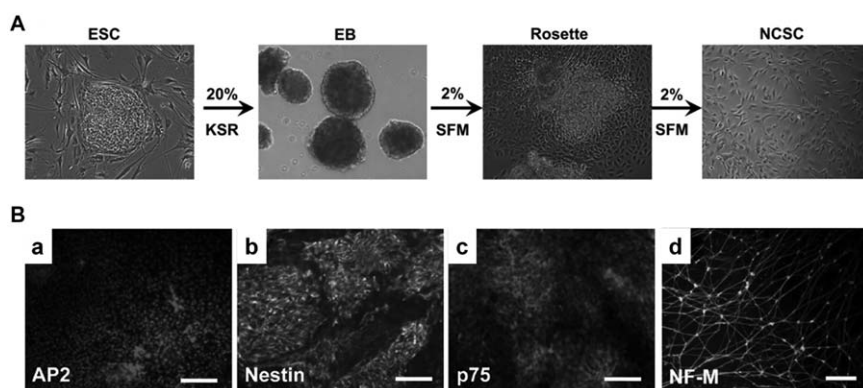


Figure 4.9 Preparation of NCSCs (neural crest stem cells) from hiPS cells and hES cells. (A) The procedure used to derive NCSCs from hiPS cells and hES cells. (B) The majority of cells were positive for AP2 (neural crest markers) (a), Nestin (b), p75 (c), and NF-M (Neurofilament M subunit) (d). Scale bars in (a), (b), and (c) indicate 100 μ m. Scale bar in d is 200 μ m.⁶⁴ Adapted from ref. 64 with permission from Elsevier, Copyright 2011.

SIRPA was extensively expressed in the cell lineage of induced cells prepared from hES cells.⁵⁹

The Type D method is a relatively unpopular method for use in the induction of hPS cells because of the additional processes of FACS, MACS, or cell sorting. However, if the purity of differentiation is relatively low or if a targeted cell type is difficult to induce, such as during induction into dopaminergic TH⁺ cells or into endoderm cells such as hepatocytes or β cells, the Type D protocol should be a valuable method for the induction of hPS cells.

Various types of human somatic cells are obtained from hPS cells through EB generation. However, heterogeneous lineages of cells are generated from the induction of hPS cells through EB generation. Especially targeted somatic cells need to be generated after the selection or purification of cells from EB using cell sorting by FACS or MACS or mechanical selection, such as that selected from rosette morphologies. The purification of cells *via* cell sorting plates or membrane filtration method having cell recognition sites, which can recognize desired cells, may be considered for the cell selection originated from EB in the future.

4.2.3 Induction of hPS Cells Seeded on Materials Directly

hPS cells are able to induce differentiation on material surfaces that do not pass through EB generation, which is similar to the differentiation protocols of adult stem cells. hPS cells are cultivated on 3D microcarriers or scaffolds, in hydrogels, and on 2D materials. There are some types of differentiation protocols of this type that are categorized as Types E–G.

4.2.3.1 Type E Differentiation of hPS Cells (2D Cultivation)

In the Type E induction protocol, hPS cells are cultivated on biomaterial, Matrigel, or MEF in feeder-free cultivation conditions and subsequently colonies of hPS cells are dissociated into small aggregates of cells or single cells. These cells are then cultivated in hydrogels, on 3D scaffolds or microcarriers, or on 2D biomaterials.^{13,22,23,39,48,66–113}

This protocol is one of the most common methods for the induction of hPS cells. This is because the spontaneous differentiation of hPS cells, which generates in the Type A–D or AB protocol using EB makes inhomogeneous populations of stem cells differentiated. On the other hand, step-by-step induction methods without the use of EB generation (Types E–H), support a more limited induction of hPS cells into specific and desired cell lineages. Type E is extensively an attractive protocol due to the easiest differentiation protocol among Types E–H.

The polyacrylate-coated plates, which were immobilized with bioactive oligopeptides derived from ECM, were developed by Melkounian and his colleagues.⁷⁰ Plates immobilized with oligopeptides obtained from bone sialoprotein and vitronectin were effective at keeping the pluripotent state of

hES cells for over 12 passages. H7 hES cells were further cultivated on plates immobilized with oligovitronection in cardiomyogenic induction medium to bring about their differentiation into cardiomyocytes.⁷⁰

Cardiac differentiation of hES cells was performed by sequential exposure of cells to activin A for 1 day, following cultivation in medium containing B27 and BMP4 for 4 days. Then, the cells were cultivated in medium containing B27 but no growth factors for an additional 14–28 days. Spontaneously beating cell morphologies could be found after 14 days of differentiation into cardiomyocytes, and the cells expressed the cardiomyocyte-specific markers α -actinin (55–65% of the cells) and Nkx2.5 (55–65% of the cells) as evaluated by flow cytometry.⁷⁰ hES cells were observed to induce differentiation sufficiently on the polyacrylate-coated plates, which were immobilized with oligovitronection.

4.2.3.2 Type E Differentiation of hPS Cells (3D Cultivation)

A 2D monolayer cultivation has several benefits over EB generation with respect to the efficient induction of hPS cells into desired lineages because 2D cultivation means that cells receive similar levels of stimuli from both neighboring cells and cultivation medium, compared with EB initiation where cells obtain a variety of signals depending on their positions within the EBs. This difference is very important for the efficient induction of DE (definitive endoderm) because high levels of the growth factor of activin A as a supplement in cultivation medium are required for the optimal differentiation of hPS cells into DE cells.¹⁰¹ However, the absence of optimal cell contacts in 2D cultivation conditions leads to restricted properties, because tissue development is also controlled by cell–cell contacts. It was found that 3D cultivation of hepatocytes could restrict the functional decrease of hepatocytes, which is generally found in 2D cultivation conditions.^{114–116}

Therefore, Ramasamy and his colleagues hypothesized that the transfer of DE cells induced from hES cells in initial 2D cultivation conditions into a 3D cultivation condition would enhance hepatocyte functionality and maturation.¹⁰¹ Using this strategy, hES cells (H1) were cultivated in a 2D monolayer in a medium including activin A and sodium butyrate for 3 days. Following this, the cells were cultivated in stage II medium containing knockout serum replacement (KOSR) and dimethyl sulfoxide for 1 day in a 2D monolayer. The cells were then seeded onto an alginate scaffold (Algimatrix plate) to proceed with 3D cultivation and were cultivated in stage II medium for 4 days. Before the inoculation, the cells were treated with the ROCK inhibitor Y-27632 for 2 h to enhance cell viability and sphere formation on the scaffolds. Then, the cells on the scaffold were cultivated in stage III medium containing FBS, tryptose phosphate broth, hydrocortisone 21-hemisuccinate, insulin, hepatocyte growth factor (HGF) and oncostatin M (Onco M) for 4–5 days to generate mature hES cell-derived hepatocytes.¹⁰¹

The gene expression patterns of hES cell-derived hepatocytes prepared from 3D cultivation were compared to the gene expression patterns from a

monolayer cultivation to investigate whether 3D cultivation on alginate scaffolds had some benefits over monolayer cultivation in the induction of DE cells into hepatocytes.¹⁰¹ Hepatocytes, which were induced from hES cell-derived DE cells in both 3D and 2D cultivation expressed hepatic genes, such as the cytochrome P450 family members CYP7A1 and CYP3A4, TO (tryptophan dioxygenase), ApoF (apolipoprotein F), and albumin. The cells induced by differentiation on the 3D alginate matrices expressed much higher levels of hepatic markers in comparison to the cells induced by differentiation on the monolayer, especially with respect to genes displayed in more mature hepatocytes, such as ApoF, TO, CYP7A1, and CYP3A.¹⁰¹

CYP3A4 activity in HepG2 cells (positive control as a human hepatic cell line) and hES cell-derived hepatocyte was studied to investigate the improvement resulting from 3D cultivation on hES cell induction into functional hepatocytes, because CYP3A4 is a critical P450 enzyme in the human liver. hES cell-derived hepatocytes generated on a 3D alginate scaffold expressed greatly enhanced levels of CYP3A4 activity compared with the 2D monolayer cultivation at a low inoculating density of 0.5×10^6 cells per well.¹⁰¹ A higher seeding density enhanced CYP3A4 activity to a lesser extent. This is explained by the suboptimal spheroid size. The cells that were cultivated on the 3D alginate scaffold also showed enhanced CYP3A4 activity in response to rifampicin addition, which was a typical function of hepatocytes.¹⁰¹ The production of urea by hES cell-derived hepatocytes generated on a 3D alginate scaffold at a low inoculating density of 0.5×10^6 cells per well was especially increased in comparison to hepatocytes generated on 2D monolayer cultivation.¹⁰¹ These results indicate that 3D cultivation of hES cell-derived DE cells in an alginate scaffold is preferable in enhancement of the maturation of hepatocytes and promotes hepatocyte function and gene expression.

4.2.3.3 Type F Differentiation of hPS Cells

In the Type F differentiation protocol, hPS cell colonies are dissociated into small aggregates of cells or single cells after hPS cell proliferation on biomaterial, Matrigel, or MEF. Then, the cells are cultivated on somatic cells or tissues that control the induction of hPS cells into the desired tissue cells.^{50,117–124}

hNCSCs (human neural crest stem cells) provide opportunities to study the development of neural crests and offer clinical treatment models of neural crest-related disorders, although it is difficult to induce hPS cells into hNCSCs.

Lee and his colleagues investigated the purification, expansion, and directed induction of hNCSCs from hES cells. hES cells were cultivated on MS-5 cells (stromal cells) in serum replacement medium including beta-mercaptoethanol for 16 days.¹¹⁷ Then, the cells were cultivated in N2 neural cultivation medium supplemented with sonic hedgehog (SHH), FGF-8, brain-derived neurotrophic factor (BDNF), and ascorbic acid (AA). Rosette morphologies were formed after 20–30 days of induction. The neural crest

marker AP2 expressed extensively in the cell clusters around the rosettes, suggesting that neural crest precursors spontaneously generated in hES cell-derived neural rosettes.¹¹⁷

The rosettes were mechanically selected and inoculated on PLO (poly-ornithine)-LN (laminin)-precoated plates in N2 medium (P1, passage 1). After 6–7 days of P1 cultivation, cells were stained with antibodies against the hNCSC marker p75 and purified by FACS. The purified hNCSCs were inoculated on PLO/LN-precoated plates and cultivated.¹¹⁷ The hNCSCs were expanded *in vitro* and differentiated toward peripheral nervous lineages (Schwann cells and peripheral neurons) and mesenchymal lineages (chondrocytes, osteoblasts, adipocytes, and smooth muscle cells [SMCs]).¹¹⁷

The interaction generating between the environment in the adult brain and hES cells remains unclear. Therefore, Tabar and his colleagues induced hES cells into neural precursor cells and studied the induction of hES cell-derived neural precursors into oligodendrocytes, astrocytes, and neurons in lesion and normal brains of young adult rats.¹¹⁸ Neural induction from hES cells was performed under serum-free conditions by co-culture on MS-5 cells (stromal cells). Neural precursors were mechanically selected and inoculated onto PLO/LN-coated plates after 30 days in cultivation.¹¹⁸

When the neural precursors were located into rat brain from striatal implants, the hES cell-derived neural precursors were observed to be merged into endogenous precursor pools in sites of persistent neurogenesis, subventricular zone. hES cell-derived precursors migrated along a rostral migratory stream to the olfactory bulb and contributed to neurogenesis.¹¹⁸

4.2.3.4 Type G Cell Differentiation of hPS Cells

In the Type G differentiation protocol, hPS cells are cultivated on materials in induction media for a few days and are subsequently dissociated into single cells after their proliferation on biomaterial, Matrigel, or MEF, and purified by mechanical selection of cells (*e.g.*, rosette structure cell selection), FACS, or MACS to be further selected into a specific cell type.^{98,117,118,121,122,125,126}

Current endothelial cell (EC) induction methods are not efficient, and the phenotypes of ECs are only briefly stable (for 14 days or less), which greatly limits their use in basic science study. Therefore, Zhang and his colleagues proposed an excellent hiPS cell-EC induction method, which incorporates 3D fibrin scaffolds.¹²⁶ In their method, hiPS cells were inoculated onto a patch of fibrin scaffolds for cultivation in a 3D environment. At the first stage of differentiation (mesoderm differentiation), hiPS cells in the fibrin matrix patch were cultured in media supplemented with activin A and BMP-4 for 1 day. At stage 2 of differentiation into ECs, hiPS cells in the fibrin matrix patch were cultured in medium with B27, VEGF, TGF- β 1, and erythropoietin (EPO) for 4 days.¹²⁶ On day 5, the differentiating hiPS cells were released by treating the patch with collagenase IV and were cultured in medium including SB-431542, VEGF, and B27. The media was changed every 2 days. On day 14, CD144- and/or CD31-expressing cells (ECs; CD144⁺ and/or CD31⁺

cells, respectively) were selected by FACS and proliferated in medium containing SB-431542, VEGF, and B27.¹²⁶ Between 25% and 46% of the differentiated hiPS cells showed an EC phenotype (CD31⁺ cells) in the fibrin matrix patch before cell selection by FACS. On the other hand, only 5% of cells showed an EC phenotype in typical monolayer cultivation in any types of hiPS cells, which were studied in this project. Over 95% of the cells in the fibrin matrix patch showed an EC phenotype after selection of CD31 cells by FACS. hiPS cell-derived ECs were observed to express EC properties continuously for 1 month *in vitro* in this study.¹²⁶

Protein and gene expression levels of vWF-8 (von Willebrand factor-8), CD144, and CD31 were observed to be extensively up-regulated in hiPS cell-derived ECs. hiPS cell-derived ECs biologically worked in the uptake of Dil-ac-LDL (Dil-conjugated acetylated low-density lipoprotein) and generated tubular morphologies on Matrigels (Figure 4.10). These data indicated that a 3D induction method with cell selection by FACS (or MACS) can extensively produce ECs from hiPS cells and that hiPS cell-derived ECs show EC function and can support EC fate for up to 1 month *in vitro*.¹²⁶

This study shows that the physical 3D surface tension, which is generated by a fibrin matrix patch, facilitates EC induction of hPS cells and entraps inducers and mimics the microenvironment of myocardium development.

Human neural stem (NS) cells that can be induced into some neural lineages are commonly generated by the isolation (a mechanical process) of rosette cells, as was discussed by Zhang *et al.*⁶³ and Wang *et al.*⁶⁴ Rosette cells are generated *via* EB formation as well as from cultivation of dissociated hPS cells from a colony of hPS cells on stromal cells (*e.g.*, MS-5).^{117,118,121,122} Following this, the rosette cells are prepared and are

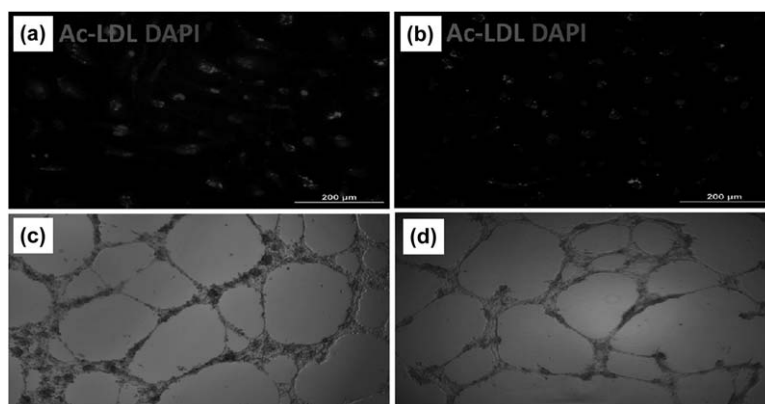


Figure 4.10 Evaluation of differentiated hiPS cell-derived endothelial cells (ECs). The biological function of hiPS cell-derived ECs was analyzed by Dil-ac-LDL uptake (a and b) and by the generation of tube-like morphologies on Matrigel (c and d) after 7 days in cultivation (a and c) and 1 month in cultivation (b and d) after isolation. Scale bars indicate 200 μm .¹²⁶ Adapted from ref. 126 with permission from Elsevier, Copyright 2014.

mechanically chosen. Then, the rosette cells are cultivated on materials such as PLO/LN-coated plates to be differentiated.

4.2.3.5 Type H Differentiation of hPS Cells

In the Type H differentiation protocol, hPS cells are transfected or transduced with some induction genes and/or antibiotic resistance genes, such as puromycin resistance genes after their propagation on biomaterial, Matrigel, or MEF. Then, cells that have induced into desired tissue cells are selected with or without antibiotic treatment.^{104,119,127,128}

Stem cells respond to genetic signals to facilitate lineage-specific induction. Zoldan and his colleagues investigated a protocol for delivering siRNA (small interfering RNA) to hES cells in a 3D cultivation matrix using lipidoids¹²⁹ (lipid-like material).¹²⁸ Through siRNA silencing of the KDR (kinase insert domain receptor) gene, the cells achieved concurrent reduction (60–90%) of genes, which was representative of ectoderm and endoderm germ layers and extensive enhancement of genes, which was representative of the mesoderm germ layer (30- to 90-fold).¹²⁸ This research indicated that siRNA controls stem cell induction by inhibiting gene expression, which is representative of some germ layers. This potential to reduce endoderm germ layer induction could lead to enhanced regulation over hES cell induction into respective cell types.

Nagamoto and his colleagues reported hiPS cell- and hES cell-derived hepatocytes as follows.¹¹⁹ (1) In mesendoderm induction, hPS cells were cultivated on Matrigel-coated plates in media including FGF-2, activin A, BSA (bovine serum albumin), sodium selenite, apotransferrin, and insulin for 2 days. (2) For DE induction, the mesendoderm cells were transduced with FOXA2 and HNF1 α genes for 1.5 h on day 6 and cultivated for 72 h on Matrigel-coated plates in hepatocyte culture media included with BMP-4 and FGF-4. (3) For the expansion of hepatoblasts, DE cells were further transduced with FOXA2 and HNF1 α for 1.5 h (second time) and cultivated for 72 h on Matrigel-coated plates in hepatocyte media including FGF-10, FGF-4, FGF-1, and HGF. (4) For hepatic maturation, the cells were cultivated on Matrigel-coated dishes for 48 h in media including dexamethasone, Oncostatin M, HGF, insulin, FBS, and tryptose phosphate broth. A cell sheet made of Swiss 3T3 fibroblasts was stratified onto the hPS cell-derived hepatocyte from day 14 for 1 day (Figure 4.11). On day 15, Matrigel was stratified onto the cells for 10 days.¹¹⁹

The amount of albumin production and gene expression of hepatic markers (such as cytochrome P450 enzyme and conjugating enzyme) in the hPS cell-derived hepatocytes that were generated from the above 3D co-culture using a cell sheet made of Swiss 3T3 fibroblasts were significantly enhanced in comparison with hPS cell-derived hepatocytes that were induced in monolayer cultivation. The results suggest that the secretion of collagen type I by Swiss 3T3 fibroblasts may play a significant part in maturation of hepatocytes.¹¹⁹ The hPS cell-derived hepatocyte, which was

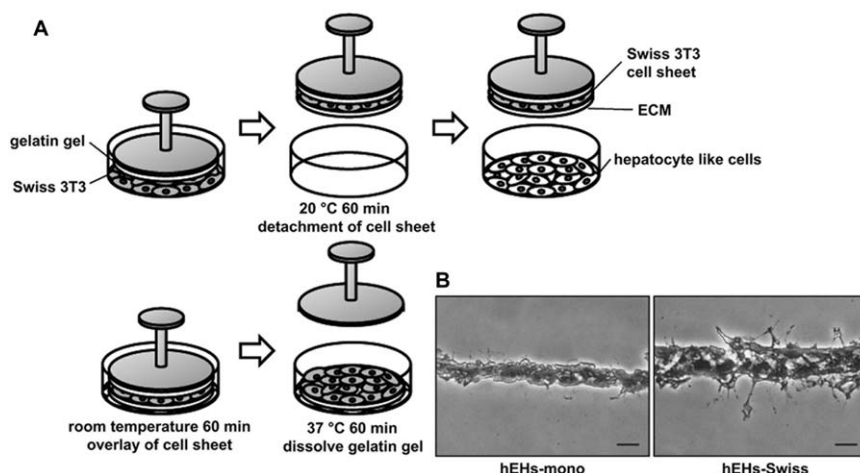


Figure 4.11 Schematic pictures of the processes used to stratify Swiss 3T3 cells on hepatocyte-like cells. (A) The stratifying method was selected by using a gelatin-coated manipulator. Swiss 3T3 cells were cultivated on a temperature-responsive cultivation plate. A gelatin-coated cell sheet manipulator was located on them, and the cultivation temperature was decreased to 20 °C for 1 h to detach the manipulator from the culture plate after the Swiss 3T3 cells reached confluence. The Swiss 3T3 cells were then harvested as a contiguous cell sheet, which was adhered on the gelatin. The Swiss 3T3 cell sheet was then stratified on hES cell- or hiPS cell-derived hepatocyte-like cells (hEHs or hiPHs, respectively). The cultivation plate with the manipulator was incubated at room temperature for 1 h to induce adhesion between the Swiss 3T3 cell sheet and the hEHs or hiPHs. To remove the gelatin, the cultivation plate was incubated at 37 °C for 1 h. (B) Phase-contrast micrograph of vertical sections of hepatocyte-like cells stratified with Swiss 3T3 cell sheet (hEHs-Swiss) and monolayer hES cell (H9)-derived hepatocyte-like cells (hEHs-mono) on day 15. Scale bars = 25 μm .¹¹⁹ Adapted from ref. 119 with permission from Elsevier, Copyright 2012.

induced by the above 3D co-culture conditions using a cell sheet made of Swiss 3T3 fibroblasts, may be valuable in clinical and research use, including drug screening for hepatocytes.

Salick and his colleagues studied cardiomyocyte induction of an engineered H9 hES cell line including a cTnT-GFP (cardiac troponin T GFP) promoter.¹⁰⁴ The cTnT-GFP promoter line also contained resistance to the antibiotic zeocin in conjunction with expression of cardiac troponin T. The promoter led to the selection of cTnT-expressing cells by zeocin treatment, leading to a 98% pure population of cardiomyocytes.¹⁰⁴ Gene delivery into hPS cells *via* transduction sometimes causes typical concerns in the gene therapy community, because it may generate genetic disorders that can cause tumor formation in patients. This protocol is currently not popular for the induction of hPS cells, although a high purity of desired cells of a desired lineage can be simply generated from hPS cells using some gene transduction.

4.3 Physical Cues of Materials in hPS Cell Induction

Stem cell fate of differentiation is influenced by some factors in the micro-environments of stem cells: (1) physical factors, such as the mechanical forces generated by cell cultivation materials, the topographies of cell cultivation materials, the elasticity (rigidity) of cell cultivation matrices (bio-materials), and the concentration of oxygen (*e.g.*, hypoxia conditions);¹³⁰ (2) cell-biomacromolecule (or biomaterial) interactions; (3) cell-cell interactions; and (4) soluble factors (Figure 4.1).^{2,7} Current studies indicate that the interactions between the physical microenvironment of stem cells and the cells are key factors for their induction fate, although the induction of stem cells commonly relies on the addition of inhibitors and soluble growth factors when no spatial regulation of the induction process is used. Physical forces are important to embryogenesis during the lineage specification of the gastrulation phase, where an embryo is transformed from spherical cells into a multilayered organism made of well-organized endoderm, mesoderm, and ectoderm germ layers.³⁴ Physical forces in cells can be generated in response to physical cues of materials, such as their topographical and/or elastic characteristics.

This chapter discusses how hPS cell fate of differentiation is influenced by (a) the mechanical forces related to materials (electrical stimulation through materials and stretching of materials) that are used for hPS cell cultivation, (b) the topography of materials that are used for hPS cell cultivation, and (c) the elasticity of materials used for hPS cell cultivation.

4.3.1 Effect of Elasticity of Cell Cultivation Biomaterials on Stem Cell Induction

A variety of microenvironmental cues affect overall stem cell fate (*i.e.*, induction into desired lineages of the cells). In particular, physical interactions between cells and the elasticity (or stiffness and rigidity) of the ECM where the cells are cultivated can contribute to stem cell fate of differentiation. However, the regulation of stem cell fate is typically considered to be generated by molecular and genetic mediators.^{131,132} At present, several investigators recognize that the stiffness of cell cultivation materials guides the lineage commitment of hMS cells (human MS cells). Stem cells trigger to induce efficient differentiation into desired tissue lineages when hMS cells are cultivated on materials with similar stiffness to those tissues. Figure 4.12 summarizes the elasticity of natural and synthetic polymeric materials and some human tissues obtained from the literature.^{16,133–139}

The elasticity of cell cultivation materials can greatly influence focal adhesions, cell phenotype, and cell morphology, particularly in 2D cultivation.^{131,140–150} Mechano-sensing of biomaterials detected by stem cells is performed by integrin-mediated focal adhesion signaling.¹⁵¹ Integrins are known as receptors relating the adhesion between ECMs and cells in cell cultivation. Integrins consist of obligate heterodimers including two

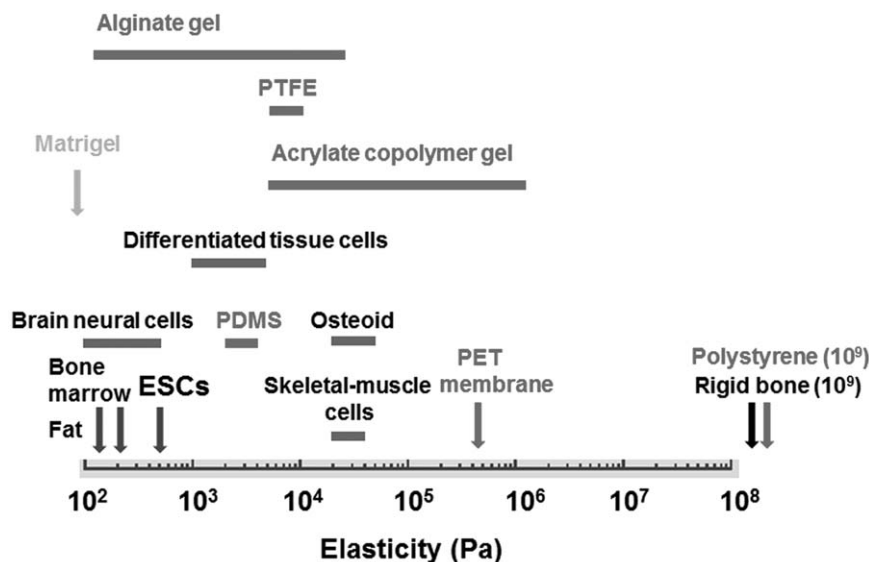


Figure 4.12 The elasticity of natural polymers, synthetic polymers, and human tissues.¹⁷

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separated chains of β and α subunits. Integrins generate cell-matrix signaling by activation of intracellular tyrosine kinase and phosphatase signaling to induce downstream biochemical signals, which are key factors for the control of stem cell fate from specific gene expression.¹³³

It is known that non-muscle myosin IIA (NMMIIA)-dependent contractility on the actin cytoskeleton is a key mediator of the mechano-transduction and mechano-sensing action in several kinds of stem cells.^{16,133,151–153} Moreover, the stiffness of cell cultivation biomaterials influences intracellular signaling *via* mechano-transducers such as FAK (focal adhesion kinase) and ROCK (Rho kinase) signaling. Subsequently the intracellular signaling controls the induction lineage of stem cells in 2D cultivation.^{142,154} We discuss the effect of biomaterial stiffness on the induction lineage of stem cells in 3D and 2D cultivation in this section. Tables 4.3 and 4.4 summarize some of the literature dealing with the effects of biomaterial stiffness on the induction of stem cells in 3D and 2D cultivation.^{16,34,138,139,141–149,155–180}

4.3.1.1 Stiffness of Biomaterials Guides Stem Cell Fate of Differentiation in 2D Cultivation

Engler and his colleagues cultivated hMS cells on PAAm (polyacrylamide) hydrogels of various stiffness immobilized with collagen type I in proliferation media (*i.e.*, culture media with no differentiation-inducing biochemical molecules).¹⁶ Figure 4.13 depicts the transcription profiles and proteins

Table 4.3 Some investigations into induction of stem cells cultivated on materials having different stiffness in 2D cultivation.¹⁷ Adapted from ref. 17 with permission from American Chemical Society, Copyright 2013.^a

Stem cell source	Materials for stem cell culture having different stiffness	Differentiation	Medium	Ref. (year)
hMSCs	HyA-gelatin-PEG hydrogels	Proliferation and secretion of cytokines	Expansion medium	169 (2009)
hMSCs	PAAm gel coated with collagen type I	Proliferation and cell morphology	Expansion medium	157 (2010)
Murine ESC (OGR1)	PAAm gel coated with collagen type I and rigid dishes coated with collagen type I	Proliferation with pluripotency	Expansion medium	148 (2010)
hMSCs	Patterned crosslinked methacrylated HyA gel containing RGDS	Proliferation and cell morphology	Expansion medium	177 (2010)
Murine ESCs (CGR8)	Polyion complex nanofilm composed of PLL and HyA	Proliferation and cell morphology	Expansion medium	170 (2010)
hESCs (H1, H9)	PDMS micropost treated by oxygen plasma	Proliferation with pluripotency	Expansion medium	171 (2012)
hMSCs	Thiol-modified HyA gels and PAAm coated with collagen type I	Proliferation and cell morphology	Expansion medium	165 (2012)
mESCs (TG2αE14)	PDMS coated with collagen type I	Proliferation, osteoblast, and mesendoderm differentiation	Expansion and differentiation medium	145 (2009)
Rat MSCs	PDMS grafted with polyacrylic acid	Osteoblast	Differentiation medium	166 (2009)
hMSCs	PAAm gel coated with collagen type I	Osteoblast	Differentiation medium	142 (2011)
hMSCs	Gelatin-hydroxyphenylpropionic acid-tyramine gels crosslinked with HRP and H ₂ O ₂	Osteoblast	Unknown	178 (2012)
Umbilical cord MSCs (Wharton's jelly)	PAAm gel grafted with collagen type I	Osteoblast	Differentiation medium	155 (2012)
Rat MSCs	PDMS coated with fibronectin and gelatin	Osteoblast	Differentiation medium	164 (2012)
hMSCs (Stro-1 enriched cells)	Polyalkyl acrylate coated with fibronectin	Osteoblast	Expansion medium	179 (2012)
hMSCs	PAAm gel coated with collagen type I and fibronectin	Osteoblast, adipocyte	Differentiation medium	139 (2009)
Human epidermal stem cells, hMSCs	PDMS and PAAm gel grafted with collagen type I	Osteoblast, adipocyte	Mixed differentiation medium of osteoblast and adipocyte	158 (2012)

hMSCs	PEG gel immobilized fibronectin	Osteoblast, adipocyte, neural cell	Differentiation medium	180 (2011)
Human placenta-derived MSCs, hADSCs	Layer-by-layer polyion complex of cationic PLL and anionic HyA	Osteoblast, adipocyte, chondrocyte	Differentiation medium	146 (2009)
Murine embryonic mesenchymal progenitor cells (C3H10T1/2)	PCL nanofibers and PCL-PES nanofiber by electrospinning method	Osteoblast, chondrocyte	Differentiation medium	172 (2011)
MSCs	PAAm gel coated with collagen type I	Osteoblast, myocyte, and neuron	Expansion medium	16 (2006)
hMSCs	PAAm gel grafted with polytrimethylphosphate, polyallylamine, polyacrylic acid, or collagen type I	Osteoblast, myocyte, neuron	Expansion and differentiation medium	149 (2011)
Murine cardiac progenitor cells, MSCs	PLLA, PCL, PLGA film	Cardiomyocyte	Differentiation medium	173 (2008)
hMSCs	PAAm gel grafted with collagen I	Adipocyte, chondrocyte, smooth muscle cell, Schwann cell	Expansion and differentiation medium	141 (2011)
hMSCs	PAAm gel grafted with collagen I	Myocyte, neural cell	Expansion medium	147 (2011)
hMSCs	Gelatin-hydroxyphenylpropionic acid gel crosslinked with HRP and H ₂ O ₂	Myocyte, neuron	Expansion medium	143 (2010)
Rat NSCs	RGD conjugated PEG-PAAm interpenetrating network gel	Neuron and astrocytes	Differentiation medium	144 (2008)
Embryonic cortices	Xyloglucan gel grafted with poly-D-lysine	Neuron	Differentiation medium	176 (2009)
Rat NSCs	Polymethacrylamide-chitosan gel coated with laminin	Neuron, oligodendrocyte, astrocyte	Differentiation medium	174 (2009)
Bovine limbal stem cells	Collagen type I gel coated with laminin	Limbal epithelial cell	Expansion medium	167 (2012)
Murine ESCs (ESD3)	Fibrin gel	Endoderm cell	Differentiation medium	175 (2012)

^aESCs, embryonic stem cells; hADSCs, human adipose-derived stem cells; hMSCs, human MSCs; HyA, hyaluronic acid; MSCs, mesenchymal stem cells; NSCs, neural stem cells; PAAm, polyacrylamide; PCL, poly(ϵ -caprolactone); PDMS, polydimethylsiloxane; PEG, polyethyleneglycol; PES, polyethersulfone; PLL, poly-L-lysine; PLLA, poly(L-lactic acid).

Table 4.4 Some investigations into induction of stem cells cultivated on materials having different stiffness in 3D cultivation.¹⁷ Adapted from ref. 17 with permission from American Chemical Society, Copyright 2013.^a

Stem cell source	Materials for stem cell culture having different stiffness	Differentiation	Medium	Ref. (year)
hMSCs	PAAm gel coated with collagen type I	Proliferation and cell morphology	Expansion medium	157 (2010)
hMSCs	Thiol-modified HyA gels and PAAm coated with collagen type I	Proliferation and cell morphology	Expansion medium	165 (2012)
Rat MSCs	Gelatin- β tricalcium phosphate sponge	Osteoblast	Differentiation medium	168 (2005)
hMSCs	PAAm gel coated with collagen type I and fibronectin	Osteoblast, adipocyte	Differentiation medium	139 (2009)
hMSCs	RGD-modified alginate gel	Osteoblast, adipocyte	Mixed differentiation medium of osteoblast and adipocyte	156 (2010)
Rat MSCs	Collagen-glycosaminoglycan scaffold	Osteoblasts, chondrocytes	Expansion medium	160 (2012)
hMSCs	Thiotrophic gel composed of PEG-silica and RGD-alginate gel	Osteoblast, myocyte, and neural cell	Expansion medium	159 (2010)
Goat MSCs	Tyramine-HyA gel crosslinked with HRP and H ₂ O ₂	Chondrocytes	Differentiation medium	161 (2012)
Murine cardiac progenitor cells, MSCs	PLLA, PCL, PLGA having hexagonal or square grid geometry	Cardiomyocyte	Differentiation medium	173 (2008)
Rat MSCs	PEG nanofiber coated with collagen type I	Smooth muscle cell, endothelial cell	Expansion medium	163 (2012)
Embryonic cortices	Xyloglucan gel grafted with poly-D-lysine	Neuron	Differentiation medium	176 (2009)
Rat NSCs	Alginate hydrogels	Neuron	Expansion medium	138 (2009)
Human fetal liver stem cells	Thiol-HyA-PEG gels	Endoderm stem/progenitor cells	Expansion medium	162 (2011)
hESCs	PLLA, PLGA, PCL coated with Matrigel	Mesoderm, endoderm, and ectoderm cell	Differentiation medium	34 (2011)
Murine ESCs (ESD3)	Fibrin gel	Endoderm cells	Differentiation medium	175 (2012)

^aESCs, embryonic stem cells; hESCs, human ESCs; hMSCs, human MSCs; HyA, hyaluronic acid; MSCs, mesenchymal stem cells; NSCs, neural stem cells; PAAm, polyacrylamide; PCL, poly(ϵ -caprolactone); PEG, polyethyleneglycol; PLGA, poly(lactic acid-co-glycolic acid); PLLA, poly(L-lactic acid).

studied by Engler and his colleagues of an osteoblast transcription factor (Runx2), a muscle transcription factor (MyD1), and neuronal markers (β -III tubulin and P-NFH, Table 4.1), expressed in hMS cells cultivated on biomaterials with varying elasticity.¹⁶ Several other transcription factors and proteins expressed in MS cells cultivated on biomaterials with varying elasticity studied by other investigators^{141,144,149,160,163} are also shown in Figure 4.13. Rigid hydrogels similar to collagenous bone induced the marker expression of early osteoblast, Runx2 at a stiffness of around 35 kPa.¹⁶ Softer hydrogels, with similar stiffness to the brain at around 0.3 kPa, tend to generate cells to express early neuronal markers and morphologies (β -III tubulin and P-NFH), whereas more rigid hydrogels around 10 kPa tend to mimic cells expressing MyoD (myogenic marker) in Engler's literature.¹⁶ C2C12 (myoblast) also showed material elasticity-dependent expression of MyoD, whereas high MyoD expression was observed in C2C12 cells cultivated on hydrogels with an elasticity around 10 kPa. However, the intensity of MyoD expression in C2C12 cells was

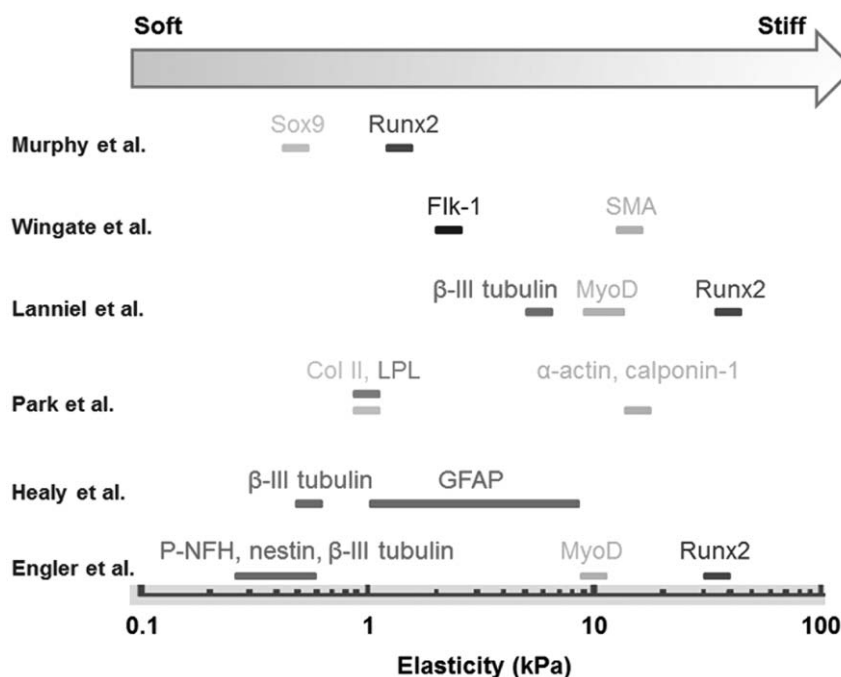


Figure 4.13 Transcription and protein profiles of an endothelial marker (Flk-1), an osteoblast transcription factor (Runx2), muscle transcription factors (SMA, calponin-1, α -actin, and MyD), an adipocyte marker (LPL), chondrocyte markers (Sox9 and collagen type II), and neural markers (β -III tubulin, P-NFH, and nestin), expressed on MS cells cultivated on biomaterials of different stiffness,¹⁷ as investigated by Engler *et al.*,¹⁶ Healy *et al.*,¹⁴⁴ Park *et al.*,¹⁴¹ Lanniel *et al.*,¹⁴⁹ Wingate *et al.*,¹⁶³ and Murphy *et al.*¹⁶⁰ Reproduced from ref. 17 with permission from American Chemical Society, Copyright 2013.

twice that of the expression in hMS cells. Furthermore, the highest expression of Runx2 (early marker of osteoblasts) was detected in hFOB (osteoblast) cultivated on hydrogels with an elasticity around 35 kPa, with an expression intensity 1.5 times higher than the expression in hMS cells.¹⁶ Rigid hydrogels facilitate focal adhesion elongation and growth. Focal adhesion supports hMS cells with force transmission pathways through which to affect their micro-environment through contraction of actin-myosin. Then, rigid cultivation biomaterials generate higher tensed and stiffer cells. Cells change their expression of non-muscle myosin to create higher forces on the actin cytoskeleton, an important point to change more rigid matrices.¹⁶ The forces formed on the actin cytoskeleton are postulated to affect stem cells induction of differentiation. Then, stem cells experience several induction fates when the stem cells are cultivated on varied cell cultivation biomaterials.^{7,131}

Some investigators further studied that the elasticity of the cell cultivation biomaterials is a valuable factor in the induction of stem cells in 2D cultivation.^{131,140–151,155,164,166,167}

The microenvironment of muscle enables primary muscle stem (MuS) cells to guide regeneration of skeletal muscle when implanted in animal models. The MuS cells cultivated on TCP dishes lose their “stemness”, which leads to progenitors with decreased regenerative ability.¹⁵⁰ Gilbert and his colleagues studied whether the elasticity modulus of cell cultivation plates plays an essential function in MuS cell function including muscle regeneration by self-renewal.¹⁵⁰ They made crosslinked PEG (polyethylene glycol) hydrogels with elasticities of 40, 10, and 2 kPa on plastic plates (1 μm thickness). As laminin (LN) is known to be one of the components of the native MuS cell niche, LN was immobilized onto the PEG hydrogel. The shortening speed of MuS cells was observed to reduce on soft PEG hydrogel (100 $\mu\text{m h}^{-1}$) in comparison with the velocity cultivated on rigid plastic cultivation plates (120 $\mu\text{m h}^{-1}$) from time-lapse observation.¹⁵⁰ The total numbers of MuS cells cultivated on rigid plastic cultivation plates were unchanged during 7 days of cultivation. This is due to the fact that cell division was offset by cell death. On the contrary, the number of MuS cells cultivated on soft PEG hydrogels was increased, to be twice that of the cells cultivated on rigid plastic cultivation plates.¹⁵⁰ This observation suggests that MuS cell cultivation on soft PEG hydrogels is able to support MuS survival. MuS cells cultivated on soft PEG hydrogels show only one-third of the expression of myogenin (transcription factor), which suggests the induction of MuS cells, than MuS cells cultivated on rigid plastic cultivation plates after 7 days of cultivation.¹⁵⁰ It was shown that soft cell culture biomaterials appear to enhance cell numbers by increasing cell viability and by inhibiting the induction of MuS cells *in vitro*. The function of MuS cells cultivated on soft and rigid cultivation biomaterials was also evaluated *in vivo* to investigate whether MuS cells cultivated on soft PEG hydrogels hold their stemness.¹⁵⁰ *In vivo* functional evaluation suggested that cultivation of MuS cells on PEG hydrogels matching the physiological modulus of muscle tissue (10 kPa) definitely retained their stemness (pluripotent state). MuS cells cultivated on PEG hydrogels having an elasticity around 10 kPa were retained in

mice after 1 month of transplantation. On the other hand, significantly decreased engraftment was found for MuS cells cultivated on rigid plastic cultivation plates.¹⁵⁰ Mice implanted with MuS cells cultivated on soft PEG hydrogels formed new myofibers originating from regeneration. An implantation evaluation of MuS cells cultivated on some biomaterials in mouse indicated that soft PEG hydrogels, but not rigid plastic cultivation plates, led to the self-renewal of MuS cells.¹⁵⁰

Healy and his colleagues studied interfacial hydrogels made by generating interpenetrating polymeric networks with a peptide including arginine-glycine-aspartic acid (RGD) sequences on the surface with elasticity ranging from 0.01 to 10.0 kPa (Figure 4.14).¹⁴⁴ Rat NS cells expanded when cultivated in serum-free medium on the RGD-grafted interpenetrating network hydrogel with elastic moduli greater than 0.1 kPa. Neural marker β -III tubulin (Table 4.1) in rat NS cells expressed the highest on the RGD-grafted interpenetrating network hydrogel having an elastic modulus of 0.5 kPa, which is close to the physiological stiffness of brain tissues.¹⁴⁴ The results suggested that neuronal differentiation probably occurred on softer RGD-grafted interpenetrating network hydrogels in mixed neuronal and glial induction media. On the other hand, the induction into glia cells was favored on stiffer RGD-grafted interpenetrating network hydrogels in the same mixed media. Moreover, differentiation, self-renewal, and cell spreading were restricted on the RGD-grafted interpenetrating network hydrogel with an elastic modulus of 0.01 kPa.¹⁴⁴ This research indicated that both biochemical (soluble biochemical factors, growth factors, ECMs, bioactive oligopeptide) and physical (elasticity of the cell cultivation materials) factors could control the desired differentiation lineages and self-renewal of rat NS cells.

Cell cultivation biomaterials with gradients of elastic modulus (or storage modulus) are interesting biomaterials used to evaluate stem cell induction regulated by substrate elasticity systematically. Some protocols for the generation of biomaterials with gradients of elastic modulus have been designed and are depicted in Figure 4.15. A monomer liquid containing a

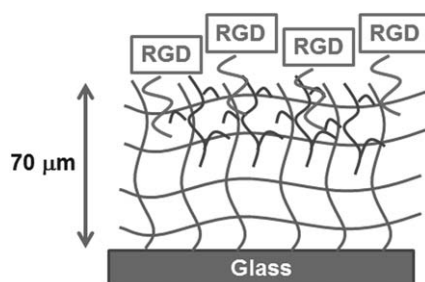


Figure 4.14 Schematic illustration reported by Healy *et al.*¹⁴⁴ of interpenetrating polymer networks with peptides containing surface RGD sequences with several stiffness.

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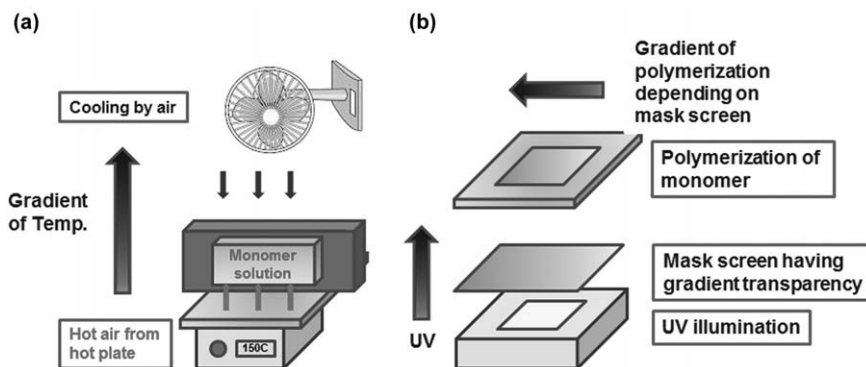


Figure 4.15 Preparation methods of biomaterials with elastic modulus gradients by monomer polymerization, made by temperature gradient (a) or intensity of UV light (b).

Part (a) adapted from ref. 164 with permission from Elsevier, Copyright 2012. Part (b) adapted from ref. 147, <https://doi.org/10.1371/journal.pone.0015978>, under the terms of the CC BY 4.0 License, <https://creativecommons.org/licenses/by/4.0/>.

crosslinker is located under UV light¹⁴⁷ (Figure 4.15B) or a temperature gradient¹⁶⁴ (Figure 4.15A), which create a gradient of crosslinking intensity and elastic modulus in the resulting biomaterials.

Tse and Engler prepared crosslinked PAAm hydrogels having radial elastic modulus gradients (1000 Pa mm^{-1}) with a range of 1000 to 14 000 Pa using photopolymerization under a gradient-patterned photomask.¹⁴⁷ hMS cells in the expansion media were found to migrate to more rigid matrices and then induced into a more contractile myogenic phenotype on the crosslinked hydrogel immobilized with collagen type I. On the other hand, hMS cells expressing β -III tubulin (neuronal marker) were retained on the soft area of the gradient hydrogel.¹⁴⁷ Some studies have suggested that soft cell cultivation biomaterials regulate MS cells into neuronal induction when MS cells are cultivated in either proliferation media or induction (differentiation) media.^{16,138,140,143,144,149,159}

TGF- β (transforming growth factor β) is reported to facilitate MS cell induction into either chondrocytes or SMCs. Park and his colleagues studied whether the stiffness of the cell cultivation biomaterials influenced the induction of hMS cells by cultivating hMS cells on crosslinked PAAm hydrogels with varying elasticity (15, 3, and 1 kPa) immobilized with collagen type I, collagen-coated cultivation plates (stiff material), and collagen type I hydrogels (soft material).¹⁴¹ hMS cells cultivated on soft hydrogels had lower proliferation rates, fewer stress fibers, and less spreading than hMS cells cultivated on stiff cultivation biomaterials. Furthermore, hMS cells on stiff biomaterials showed higher expression of α -actin and calponin-1 (SMC markers) in proliferation media. On the other hand, hMS cells on soft biomaterials showed enhanced expression of collagen type II (chondrogenic marker) and lipoprotein lipase, LPL (adipogenic marker) (Figure 4.13).¹⁴¹

The addition of TGF- β in the cultivation media reduced the expression of adipogenic markers and facilitated the expression of SMC markers on soft cultivation biomaterials. However, it should be noted that hMS cells were able to differentiate into adipocytes on soft cultivation biomaterials when hMS cells were cultivated in adipogenic induction media.¹⁴¹

Lanniel and his colleagues studied differentiation lineages of hMS cells cultivated on crosslinked PAAm hydrogels having several functional groups (acrylic acid, allylamine, and trimethylphosphate) and varying stiffness. Vinyl monomer was polymerized on the hydrogel surface using plasma polymerization techniques. hMS cells were cultivated on the hydrogel to investigate the effect of several combinations of chemical cues (several functional sites) and physical cues (biomaterial stiffness) on hMS cell induction.¹⁴⁹ Runx2 expression (osteogenic marker) was the highest in hMS cells cultivated in proliferation media containing no differentiation (induction) molecules on PAAm hydrogels immobilized with phosphate polymer and with a stiffness of 4100 Pa. MyoD1 (myogenic phenotype marker) was extensively expressed in hMS cells cultivated on PAAm hydrogel immobilized with polyacrylic acid with intermediate stiffness (10 000–17 000 Pa) (Figure 4.13).¹⁴⁹ Neurogenic induction as evaluated by β -III tubulin expression was the greatest on the softest hydrogel (6500 Pa) immobilized with polyacrylic acid (Figure 4.13). Matrix calcification and bone nodule formation were found on PAAm hydrogels stiffer than 10 000 Pa, which were immobilized with polyallylamine in osteogenic differentiation media but not on hydrogels immobilized with collagen type I.¹⁴⁹ These observations suggest that hMS cell differentiation lineage is controlled by not only the stiffness (elasticity) of cell cultivation biomaterials but also by surface chemistry (different kinds of functional sites) and differentiation induction factors.

4.3.1.2 *Pluripotent Maintenance of MS, iPS, and ES Cells on Soft Biomaterials*

It is necessary to keep iPS and ES cells in an undifferentiated condition for expansion of the cells in cultivation. Some researchers have reported that undifferentiated ES cell cultivation indeed includes inhomogeneous populations verified by the fluctuating expression of several cell-surface markers and transcripts.^{148,181–183} One of the big challenges in the field is to create optimal cultivation biomaterials and conditions to retain the pluripotency and self-renewal of hiPS and hES cells.

Chowdhury and his colleagues investigated whether mES (mouse ES) cells could retain their pluripotent state (as evaluated by the expression of high levels of pluripotent proteins and genes (Nanog and Oct3/4) and by the formation of homogeneous pluripotent colonies) when mES cells were cultivated in the media without exogenous LIF (leukemia inhibitory factor) on soft biomaterials (600 Pa) that match the intrinsic elasticity of mES cells. On the other hand, mES cells did not retain their pluripotent state and self-renewal potential on normal stiff TCP plates (>4000 kPa) immobilized

with collagen type I or on hydrogels with much stiffer moduli.¹⁴⁸ In general, it is required to input LIF into the cultivation media during the proliferation of mES cells to retain their pluripotent state and self-renewal.²

However, several lines of mES cells could be cultivated on soft biomaterials with no addition of LIF into the cultivation media, keeping the formation of homogenous pluripotent colonies with high expression of alkaline phosphatase (ALP) activity and pluripotent markers (Oct3/4) (index of pluripotent state, see Table 4.1) for up to 15 passages, indicating that their soft hydrogel could be used for long-term cultivation of mES cells.¹⁴⁸ It should be mentioned that vitronectin (VN) or LN are better ECMs compared to the collagen that was used in this research, for ECM immobilized in the cell cultivation biomaterials to retain pluripotent state and self-renewal of iPS and ES cells.² Then, it is interesting that mES cells could be cultivated on soft hydrogels immobilized with collagen type I while maintaining their pluripotent state and self-renewal for more than 10 passages in the cultivation media without LIF.¹⁴⁸ mES cell colonies on soft biomaterials in cultivation media containing no LIF had low stiffness and formed low cell-matrix traction. Both stiffness and traction of the colonies enhanced with increasing stiffness of cell cultivation biomaterials, which was also accompanied by reduced expression of Oct3/4 (pluripotent marker). This result indicates that the pluripotency and self-renewal of mES cells are retained on soft cell cultivation biomaterials *via* the biophysical mechanism of promoting the formation of low cell-matrix traction.¹⁴⁸

There is a contradictory study which found that rigid (stiff) biomaterials can maintain the pluripotent state of hES cells.¹⁷¹ Sun and his colleagues developed micropost arrays of elastomeric PDMS (polydimethylsiloxane), where the height of the PDMS microposts regulates the elasticity of cell cultivation biomaterials (elasticity) (Figure 4.16).¹⁷¹ It is reported that PDMS micropost arrays have an effect on stem cell differentiation, cytoskeleton contractility, focal adhesions, and cell morphology.^{171,184,185} Human ES cells were cultivated on micropost arrays with oxygen plasma treatment that were immobilized with VN. Human ES cells were mechano-sensitive and enhanced their cytoskeleton contractility with increase of matrix stiffness, and stiff

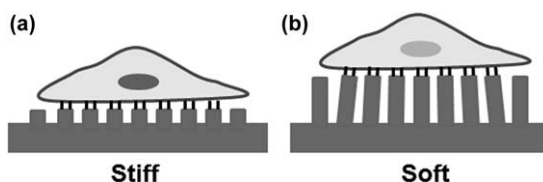


Figure 4.16 Elastomeric PDMS micropost arrays where the height of PDMS microposts regulate substrate elasticity (stiffness). Long and short microposts make soft and stiff surfaces, respectively. Both micropost arrays are composed of the same components and crosslinking of PDMS.¹⁷¹ Adapted from ref. 171, <https://doi.org/10.1371/journal.pone.0037178>, under the terms of the CC BY 4.0 License, <https://creativecommons.org/licenses/by/4.0/>.

biomaterials could retain the pluripotent state of hES cells.¹⁷¹ Matrix mechanics-mediated cytoskeleton contractility is found to be functionally related to the expression of E-cadherin in cell–cell attachment and involved in hES cell fate decision. The microenvironment of hES cell cultivation on micropost array might be different from typical 2D cultivation biomaterials.¹⁷¹ This discrepancy might lead to different optimal stiffness to keep the pluripotent state of hES cells, where rigid biomaterials are adequate in micropost array cultivation and soft hydrogels are optimal in conventional 2D cultivation.

The microenvironment of hMS cells *in vivo* controls their differentiation and self-renewal potential. Human MS cells cultivated *ex vivo* gradually decrease their pluripotent state after 8–18 passages, as evaluated by a lack of differentiation and expansion abilities. Winer and his colleagues cultivated hMS cells on 0.25 kPa crosslinked PAAm hydrogel immobilized with ECMs (collagen type I and fibronectin). These hydrogels mimic the stiffness of fat tissues and bone marrow (0.2 kPa of storage modulus for bovine bone marrow) (Figure 4.12).¹³⁹ Human MS cells cultivated on a soft hydrogel halted progression of the cell cycle, despite the presence of serum; these non-expansion hMS cells re-proliferated when located back on a stiff biomaterial. Non-proliferative hMS cells on 0.25 kPa PAAm hydrogel also showed the potential to induce into osteoblasts when cultivated on a stiff biomaterial in osteoblast differentiation media and to induce into adipocytes when cultivated on biomaterials in adipogenic differentiation media. These results indicated that hMS cells on soft (0.25 kPa) PAAm hydrogel are quiescent but competent to recover expansion or initiate terminal differentiation when cultured with optimal cues. These results indicate that the physical cue of ECM stiffness is a factor making the bone marrow niche to retain storage of hMS cells for a long time.¹³⁹

4.3.1.3 Mechanism of Stem Cell Induction by Substrate Elasticity and ECM in 2D Cultivation

The mechanism by which the stiffness of an ECM on cultivation biomaterials guides lineage specification of stem cell induction is currently not easy to elucidate. Figure 4.17 demonstrates several mechano-sensing models developed by several investigators for evaluating biomaterial elasticity and the direction of MS cell lineages of differentiation.^{140,142,186} The stiffness of the ECMs on the cell culture biomaterials creates mechanical stimuli on the plated stem cells, thereby generating changes in the remodeling and activity of FA (focal adhesion) protein.^{140–143} The elongation of FA changes depending on the stiffness of the cultivation biomaterials, indicating that ECM stiffness controls FA assembly (Figure 4.17). Integrins are the most common mechanical sensors located at the starting point of the sensing pathway.¹⁴⁰ Du and his colleagues demonstrated that the activation of $\beta 1$ integrin in MS cells was generated by soft cultivation biomaterials to a much higher degree than by stiff cultivation biomaterials.¹⁴⁰ On the other hand, the density of cell-surface integrins in MS

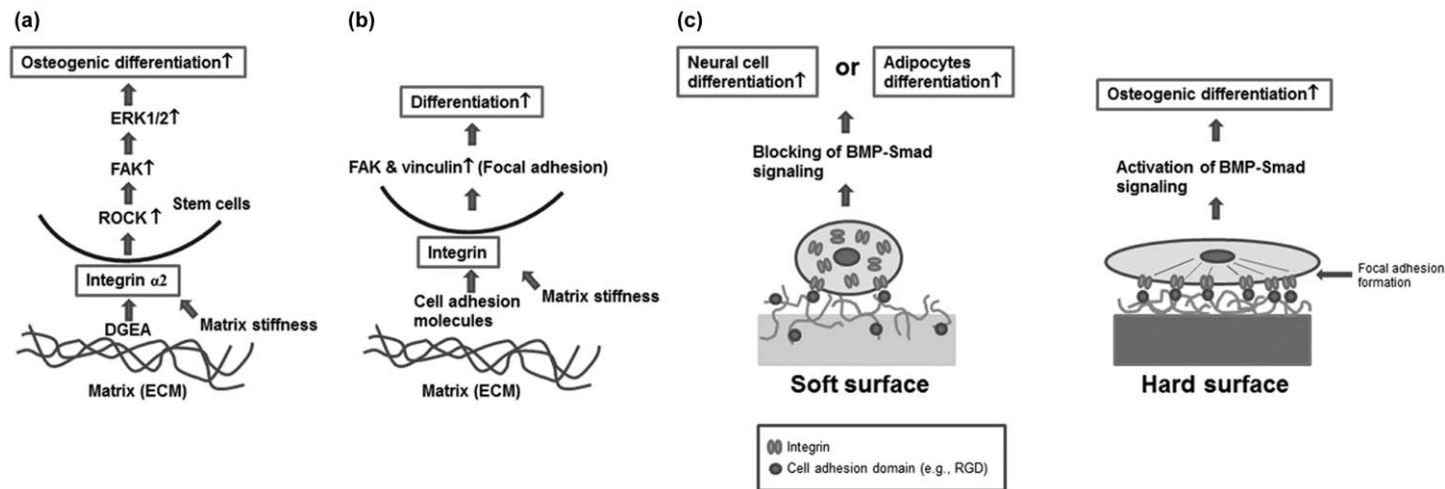


Figure 4.17 The signal transduction of the effect of culture material elasticity on stem cell induction by the growth and elongation of focal adhesion,¹⁷ as suggested by Shih *et al.* (a),¹⁴² Yim *et al.* (b),¹⁸⁶ and Du *et al.* (c).¹⁴⁰ Reproduced from ref. 17 with permission from American Chemical Society, Copyright 2013.

cells on soft cultivation biomaterials was much less than the density on rigid cultivation biomaterials in 2D conditions (Figure 4.17). Soft biomaterials markedly increased the integrin internalization; caveolae/raft-dependent endocytosis is the main cause of the integrin internalization.¹⁴⁰ The increased integrin internalization in MS cells on soft cultivation biomaterials regulates neural induction of MS cells by restricting the BMP-Smad pathway. The reduction of integrin internalization by methyl- β -cyclodextrin (caveolae/raft inhibitor) could suppress neural lineage induction of MS cells on soft biomaterials in 2D cultivation. By integrin-regulated BMP receptor endocytosis, soft biomaterials partially regulate suppression of the BMP (bone morphogenetic protein)-Smad pathway.¹⁴⁰

Atomic force microscopy (AFM) results demonstrated that integrin-receptor complexes can be more simply ruptured on soft cultivation biomaterials than on rigid cultivation biomaterials. This result might lead to an increase of integrin internalization on soft cultivation biomaterials. It is demonstrated that ECM stiffness influences the integrin activities of MS cells and trafficking mechanism regulating integrin-receptor internalization, then leading to the optimal lineage induction of stem cells cultivated on ECMs with appropriate stiffness in 2D cultivation.¹⁴⁰

4.3.1.4 Biomaterial Stiffness Guides Stem Cell Fate of Differentiation in 3D Cultivation

Stiffness of the scaffold is another valuable point for specific lineage induction of stem cells in 3D cultivation.¹⁶⁰ Some investigators have described how the stiffness of the cell cultivation scaffold (matrix) influences the differentiation lineages of stem cells in 3D cultivation.^{34,138,159–163,165}

Murphy and his colleagues developed crosslinked collagen-glycosaminoglycan scaffolds as analogues of natural ECMs. Two types of glycosaminoglycans, HyA (hyaluronic acid) and CDS (chondroitin sulfate) were used.¹⁶⁰ Crosslinking with EDC (1-ethyl-3-(3-dimethyl aminopropyl carbodiimide) and DHT (dehydrothermal) generated three glycosaminoglycan-collagen scaffolds having elasticities of 500, 1000, and 1500 Pa. In this study, the effect of glycosaminoglycan composition and scaffold elasticity on the differentiation of rat MS cells was studied in proliferation media in the absence of supplements for differentiation (induction).¹⁶⁰ The scaffold composed of HyA and with the lowest stiffness (500 Pa) promoted an extensive increase in expression of Sox9, suggesting that MS cells were induced towards the early stages of chondrocyte lineage. On the other hand, the expression of Runx2 was the highest in the most rigid (1500 Pa) scaffold composed of CDS, suggesting that MS cells were differentiated towards the early stage of osteoblasts in the most rigid scaffold.¹⁶⁰ This research shows that the stiffness of scaffolds is able to regulate the differentiation fate of MS cells in 3D cultivation even in the medium without induction supplements; this potential is further increased by the choice of appropriate components of scaffolds for desired differentiation lineages.

Toh and his colleagues developed biodegradable and injectable hydrogels made of Tyr-HyA (hyaluronic acid-tyramine) conjugates.¹⁶¹ Tyr-HyA was crosslinked *in vivo* by adding H₂O₂ (hydrogen peroxide) and peroxidase with independent tuning of the degree of crosslinking and gelation rate, both of which affected the elasticity of hydrogels.¹⁶¹ Tyr-HyA hydrogels of different stiffnesses (5400, 9500, and 11 800 Pa) were used as hydrogels for MS cells in tissue engineering of cartilage where the degradation rate, water swelling, and compressive modulus were regulated by changing H₂O₂ concentration in the oxidative coupling synthesis (Figure 4.18).^{161,187} Cellular condensation, which was evaluated by detecting the enhancement in the effective numbers of round cells in the lacunae, was observed to increase in softer hydrogels with less crosslinking density, which showed increased scaffold contracture. On the other hand, cells showed much elongated morphologies with a decreased degree of cellular condensation in much more highly crosslinked hydrogels.¹⁶¹ The density of hydrogel crosslinking regulated

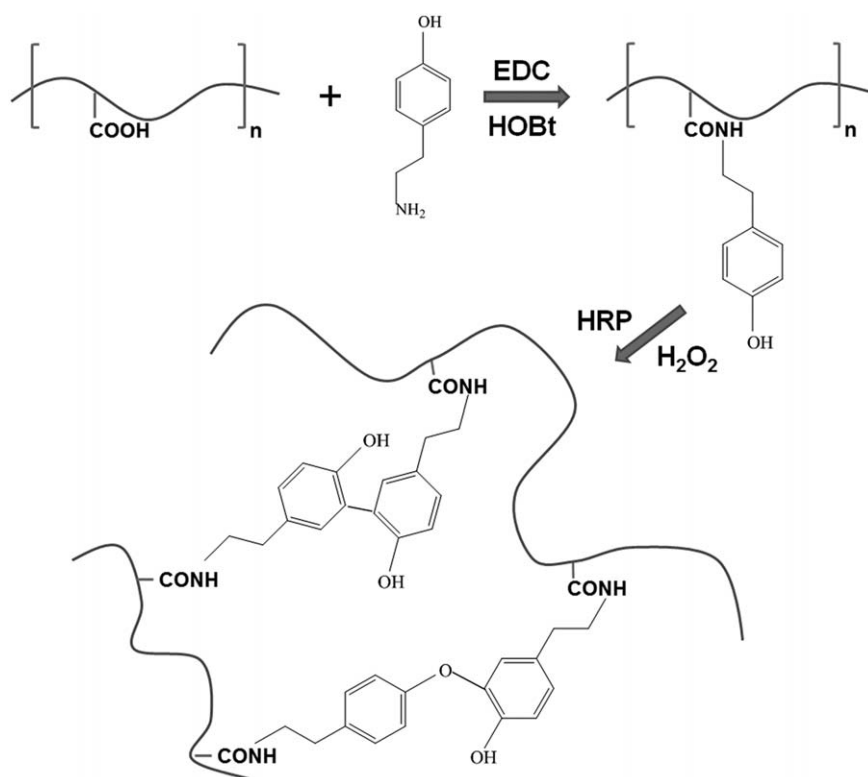


Figure 4.18 Preparation of hyaluronic acid-tyramine (HyA-Tyr) hydrogels in which their stiffness was regulated by different H₂O₂ concentration as the oxidant in the oxidative coupling reaction with HRP (horseradish peroxidase).¹⁸⁷ Reproduced from ref. 187 with permission from the Royal Society of Chemistry.

cartilage tissue histogenesis and ECM biosynthesis. Hydrogels with less crosslinking density support chondrogenic differentiation, indicating enhancement of the cells having chondrocyte morphologies and enhances the synthetic speed of collagen type II and glycosaminoglycan where hyaline cartilage tissue generation was found.¹⁶¹ This is because the tunable 3D microenvironment of the Tyr-HyA hydrogel regulates cellular condensation during chondrogenic differentiation and has an impact on matrix biosynthesis and spatial organization of cells.

Pek and his colleagues developed thixotropic silica-PEG (polyethylene glycol-silica) nanocomposite hydrogels with and without the cell adhesive RGD peptide for 3D cell cultivation.¹⁵⁹ The thixotropic silica-PEG hydrogels can be liquefied by simple application of a shear stress. This enables stem cells in the thixotropic hydrogel to be simply injected into defect sites in patient bodies before the hydrogel changes to a gel. The matrix elasticity of the hydrogels can be regulated by tuning the content of fumed silica in the hydrogel. Hydrogels were prepared with 100, 75, 40, 25, and 7 Pa of liquefaction stress, where the liquefaction stress indicates the lowest value of shear stress where the hydrogel is in a solution state on the condition of loss modulus (G'') = storage modulus (G') indicating $\tan^{-1} (G''/G') = 45^\circ$.¹⁵⁹ When hMS cells were cultivated in silica-PEG nanocomposite hydrogels in proliferation media for 7 days, the highest expression of ENO2 (neural marker) was detected in hMS cells cultivated in the hydrogel with the minimum liquefaction stress (7.0 Pa).¹⁵⁹ Moreover, the highest expression intensities of osteocalcin and Runx2 (osteoblast transcription markers) and MYOG (myogenic transcription marker) were found in hMS cells cultivated in high liquefaction stress (100 and 75 Pa) and intermediate (25 Pa) hydrogels, respectively. All of the markers were detected more significantly in hMS cells cultivated in the silica-PEG nanocomposite hydrogels than in hMS cells cultivated on 2D TCP dishes. These results suggest that hMS cells cultivated in 3D conditions are in a more differentiated condition compared to cells cultivated in 2D conditions. Grafting of the cell adhesive RGD peptide facilitated both differentiation and expansion of hMS cells on the stiffer hydrogel (liquefaction stress >75.0 Pa).¹⁵⁹ It was suggested that biomaterial elasticity controls the induction fate of hMS cells when cultivated in 3D silica-PEG nanocomposite hydrogels in proliferation media. In this study, it was shown that mechanical signal alone is able to regulate lineage specification of hMS cells in 3D cultivation. It was indicated that the mechanism by which RGD facilitates osteogenic differentiation is through its function as a ligand to the mechano-receptor of integrins, guiding stem cells to sense high matrix elasticity for osteogenic induction.

Banerjee and his colleagues investigated the differentiation and expansion of rat NS cells entrapped within 3D scaffolds of alginate hydrogels having elastic moduli ranging from 180 to 20 000 Pa made by regulating the concentration of Ca ions and alginate.¹³⁸ The speed of NS cell expansion was reduced with enhancement of the hydrogel elastic modulus in proliferation media with no added differentiation supplements. Increased expression of

β -tubulin III (neuronal marker) was detected within the softest hydrogel, which retained an elastic modulus comparable to that of brain tissues, around 180 Pa.¹³⁸ NS cells cultivated on soft hydrogels were regulated to be induced into neuronal cell lineages in 3D¹³⁸ and 2D cultivation.¹⁴⁴

Engler and his colleagues demonstrated that a cytoskeletal motor might be included in the matrices-stiffness sensor, which guides lineage determination in MS cells cultured on hydrogel.¹⁶ It would be valuable to identify an appropriate function for the cytoskeletal motor in affecting the role of NS cells in 3D culture conditions.

Some researchers have investigated the effects of cell cultivation bio-material stiffness on the induction of MS cells into vascular cell types.^{163,188–190} Wingate and his colleagues produced a 3D PEG-based nanofiber hydrogel immobilized with collagen type I with tunable stiffness for use as a cellular biomaterial guiding MS cells into vascular cells. These hydrogels are made using photopolymerization techniques and electro-spinning, and their stiffness is controlled by regulating the time of photopolymerization.¹⁶³ The elastic modulus of the hydrogel was measured by compression assay to be in the range of 2000–15 000 Pa, similar to the stiffness of the intima media layer and basement membrane *in vivo* where ECs are reported to be located on SMCs in the stiffer medial layer and top of the soft basement membranes.^{191,192} MS cells inoculated on stiff hydrogels (8000–15 000 Pa) demonstrated an enhancement of cell area compared with the cells inoculated on soft hydrogels (2000–5000 Pa).¹⁶³ It was observed that the hydrogel stiffness regulated MS cells to show several vascular-specific phenotypes with high induction ratio. 95% of MS cells cultivated on hydrogels with an elasticity of 3000 Pa showed Flk-1 (endothelial marker) expression within 1 day in proliferation media. On the other hand, only 20% of MS cells inoculated on hydrogels having elasticities >8000 Pa showed the Flk-1 expression.¹⁶³ Furthermore, 80% of MS cells cultivated on hydrogel with elasticities >8000 Pa showed α -actin (SMC marker) expression within 1 day in proliferation media, whereas fewer than 10% of MS cells inoculated on hydrogel with elasticities <5000 Pa showed the α -actin marker expression.¹⁶³ In short, the local elasticities of hydrogel-entrapped MS cells are able to regulate MS cell fate of differentiation into vascular cell lineages in proliferation media with no addition of induction supplements, and the lineage fate of MS cells into several vascular cell types are able to be regulated by the specific design of the hydrogel modulus.

Mechanical force is important to embryogenesis in the lineage determination of the gastrulation phase, where the embryo is generated from a spherical cell to a multilayered organism with properly regulated ectoderm, mesoderm, and endoderm germ layers. Zoldan and his colleagues studied the germ layer generation process by cultivation of hES cells on 3D scaffold with stiffness leading to appropriate germ layers to investigate the cell changes of the embryo in the gastrulation phase by cell environment.³⁴ The biomaterials of the scaffolds used in their investigation were PEG, PCL [poly(ϵ -caprolactone)], PLGA [poly(lactic acid-co-glycolic acid)], and PLLA

[poly(L-lactic acid)]. These scaffolds were made using the salt-leaching method.⁷ Ternary PCL/PLGA/PLLA scaffolds and binary PLGA/PLLA were also made with selected weight ratios of the biodegradable polymers. The elastic moduli of these scaffolds were observed from 50 kPa to 7000 kPa. The physical properties of each of the scaffolds are shown in Table 4.5.³⁴ Human ES cells were blended with Matrigel solution and inoculated into the scaffolds to promote cell adhesion.³⁴ Figure 4.19 depicts the expression of three germ layer-derived genes in hES cells cultivated on the scaffolds after EB generation using a Type A method (Figure 4.3) and shows relative values compared to gene expression in EBs cultivated in suspension over the same time interval.³⁴

Human ES cells cultivated on the stiffest scaffold (>6000 kPa) remained undifferentiated and showed decreased expression of the germ layer-specific genes analyzed in their research.³⁴ On the other hand, scaffolds with lowest elastic moduli (<100 kPa) demonstrated ectoderm induction, as evaluated by high expression levels of ZIC1 and SOX1 genes (ectodermal germ layer-related genes).³⁴ Scaffolds with intermediate elastic moduli (100–1000 kPa) facilitated endoderm induction and decreased mesoderm-related gene expression (MIXL1 and Brachyury). Scaffolds with medium–high elastic moduli facilitated mesodermal induction, and ectoderm- and endoderm-related gene expression were not observed.³⁴ In short, the induction of hES cells into each germ layer was facilitated by distinct scaffold stiffness thresholds, reminiscent of the forces played in the process of gastrulation. It is reasonable to consider that 3D scaffolds can dictate the mechanical stimuli demanded for guiding hES cell induction, which depends on the elasticity of the scaffold.

The liver is one of the most highly complicated tissues in our bodies and the liver's function is synthesis of serum proteins, regulation of nutrients, production of bile and hormones, and toxin removal.¹⁶² Hepatocyte stem cells work together *in vivo* with mesenchymal precursors to stellate cells and endothelia (angioblasts), and hepatocyte stem cells are located in controlled

Table 4.5 Physical and chemical characteristics of scaffolds.³⁴ Adapted from ref. 34 with permission from American Chemical Society, Copyright 2013.

Scaffold name	Component	Content	Porosity (%)	Elastic modulus (MPa)
PLGA	PLGA	100%	90	0.06
25/75	PLLA/PLGA	25%/75%	87	0.81
50/50	PLLA/PLGA	50%/50%	87	0.95
75/25	PLLA/PLGA	75%/25%	90	1.75
PLLA	PLLA	100%	86	2.22
PCL20	[PLLA/PLGA]/PCL	[40%/40%]/20%	85	0.73
PCL50	[PLLA/PLGA]/PCL	[25%/25%]/50%	87	
PCL80	[PLLA/PLGA]/PCL	[10%/10%]/80%	87	0.05
PEGDA 1% BP	[PLLA/PLGA]/PEGDA	[37.5%/37.5%]/25%	88	6.87
PEGDA 0.05% BP	[PLLA/PLGA]/PEGDA	[37.5%/37.5%]/25%	85	6.55

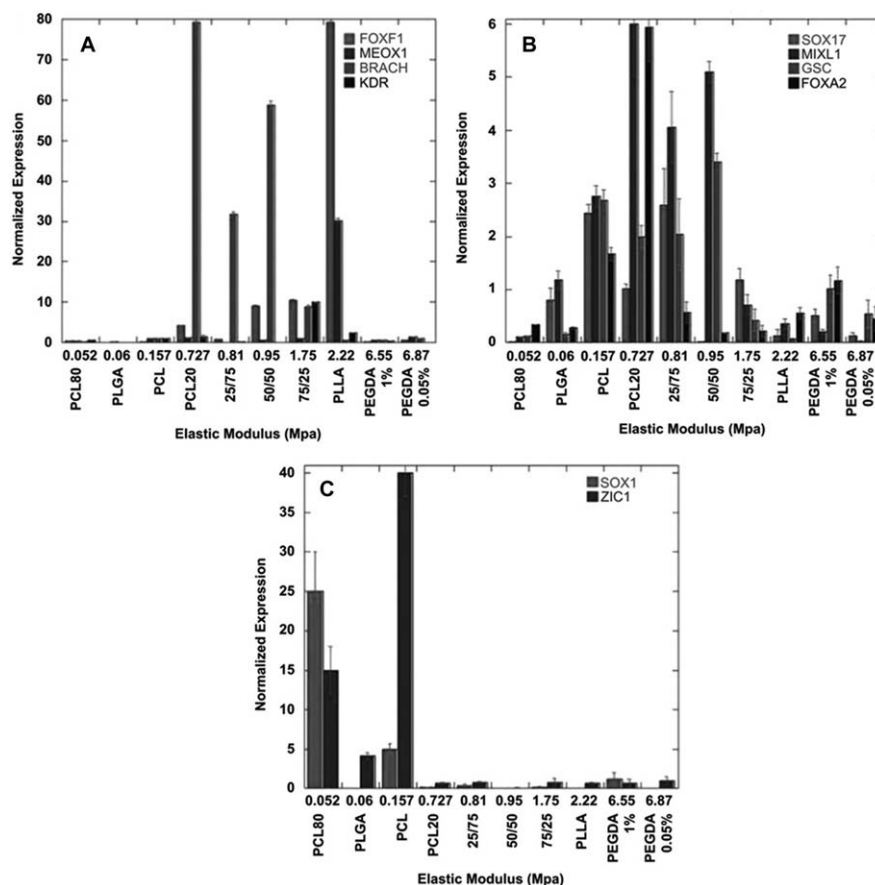


Figure 4.19 Effect of scaffold elasticity on hES cell induction. The gene expression of representative mesoderm (A), endoderm (B), and ectoderm (C) germ layers in hES cells cultivated on biomaterials after 2 weeks in cultivation was evaluated by RT-PCR and normalized against GAPDH (housekeeping gene) expression levels. Ratios of normalized values relative to their expression levels in suspended EBs were analyzed. PCL20 shows scaffolds prepared using 20% PLLA/PLGA (1:1) and 80% PCL. PCL80 means scaffolds prepared using 80% PLLA/PLGA (1:1) and 20% PCL. Values of 75/25, 50/50, and 25/75 indicate PLLA/PLGA scaffolds prepared using 75, 50, and 25% PLLA, respectively. PEGDA 0.05% and PEGDA 1% indicate scaffolds prepared using 75% PLLA/PLGA (1:1) and 25% PEGDA that were respectively crosslinked with 0.05% or 1% benzoyl peroxide.³⁴ Adapted from ref. 34 with permission from Elsevier, copyright 2011.

microenvironments with chondroitin sulfate proteoglycans, collagen type III, LN-5, and HyA. Lozoya and his colleagues studied the effects of 3D microenvironments on human hepatocyte stem cells by entrapping the hepatocyte stem cells into HyA-derived hydrogels made by a serum-free media targeted for endodermal stem/progenitor cells by resembling the liver stem cell

niche.¹⁶² These HyA-derived hydrogels fitted to the diffusivity of cultivation media and had regulated stiffness (0.025–0.52 kPa), which depended on their concentration of crosslinker (polyethylene glycol diacrylate) and HyA. The HyA-derived hydrogels guided the stem cells into the transition of hepatocyte stem cell colonies towards stable heterogeneous populations of hepatocyte progenitors (hepatoblasts), which depend on hydrogel stiffness.¹⁶² This research shows that the mechanical properties of the stem cell niche are able to control induction into endodermal stem cell populations.

The induction of hES cells into each of the germ layer phenotypes was observed to be promoted by a specific stiffness threshold in each of the scaffolds, which was reminiscent of the forces worked during the gastrulation processes. The results indicate that 3D scaffolds can sense the mechanical stimulus, which is necessary for guiding hES cell induction, and that the stimulus contributes a key function in hPS cell differentiation fate in 3D scaffolds.

4.3.1.5 Results Contradictory to Engler's Study in 2D Cultivation

Engler and his colleagues¹⁶ reported a landmark work, demonstrating that biomaterial matrices guide stem cell fate of differentiation (6500 citations by Web of Science in November 2018), although the mechanism by which stem cells sense the geometrical and mechanical characteristics of the biomaterials remains elusive.¹⁹³ Several investigators have suggested various intriguing ideas and conflicting results on the effect of biomaterial stiffness on stem cell fate of differentiation.^{156,158}

Trappmann and his colleagues studied the differentiation of human epidermal stem cells and hMS cells on (a) PAAm hydrogels immobilized with collagen type I and (b) PDMS immobilized with collagen type I.¹⁵⁸ PAAm hydrogels and PDMS of different stiffness in the range of 100–2300 kPa were generated by regulating the ratio of crosslinker to base monomers. It was observed that epidermal stem cells showed widely spread morphologies and assemble a cortical F-actin cytoskeleton on any PDMS surface, which does not depend on the stiffness. Moreover, cells cultivated on any PDMS surface showed the cornified envelope precursor involucrin at the same rate around 25% and reached terminal differentiation of epidermal stem cells (*i.e.*, keratinocytes). Therefore, PDMS stiffness did not influence the induction of human epidermal stem cells in any way.¹⁵⁸ Moreover, osteogenic induction, as evaluated by ALP activity (Table 4.2), was found with the same percentage (around 35%) on any PDMS surface of different stiffness in the range of 100–800 000 Pa. No effect of PDMS elasticity on the induction of hMS cells into osteoblasts was found. hMS cell differentiation into adipocytes was also evaluated on PDMS surfaces of different elasticities, which was analyzed by Oil Red O staining (Table 4.2). As was observed in osteogenesis of hMS cells on PDMS surfaces, no effect of PDMS elasticity was evaluated on the induction of hMS cells into adipocytes. The phenomenon is elucidated by

the properties and morphologies of the ECMs (collagen I in this case). It is suggested that ECMs should be the same on PDMS surfaces of different elasticities because ECMs do not penetrate through the PDMS surface. On the other hand, ECMs penetrate through the surface of hydrogels, as described in Figure 4.20.

Other investigators also discussed that there were no effects of PDMS stiffness on the induction of hMS cells into several types of cells.¹⁶⁴ On the other hand, cell area of adhesion and actin synthesis of hMS cells cultivated on PAAm hydrogels immobilized with collagen type I was enhanced with increased elastic modulus of the PAAm hydrogel, which was the same trend examined by some investigators in 2D cultivation of hMS cells on PAAm hydrogels coated with collagen type I.¹⁶ In short, the induction of hMS cells is affected by the elasticity of PAAm hydrogels but not the PDMS surface.¹⁵⁸

More rigid PAAm hydrogels have larger crosslinking spots. In this case, crosslinking differences in the PAAm hydrogel network are based on the differences in collagen adhesion, the distance between covalent anchoring sites being shorter on stiffer hydrogels. On the other hand, the anchoring point distance is longer on softer hydrogels, as depicted in Figure 4.20. ECMs are able to anchor into softer hydrogels with fewer anchoring sites, while ECMs anchor into stiffer hydrogels with more anchoring sites. The concentration of ECMs on the outer surface is lower on softer hydrogels and higher on stiffer hydrogels, while the total ECM amount on the hydrogel remains unchanged using evaluation of fluorescent probe binding to the ECM.^{16,142} Then, the depth of ECM anchoring is speculated to be deeper on softer hydrogels, as shown in Figure 4.20. The ECM anchoring depth should be another key point controlling stem cell fate of induction.

When stem cells pull on covalently adhered collagen, the mechanical feedback is made up of the magnitude of the movement of the collagen segments bound to the PAAm hydrogels. Then, the intensity of the collagen stiffness feedback, which cells dictate upon integrin ligation, increases with decreasing anchored collagen fiber length (for example, on stiff PAAm hydrogels).^{158,193} On the other hand, collagen elasticity on PDMS surfaces with different elasticity is considered to be equal because of the same collagen crosslinking time guiding the similar collagen crosslinking distance. This is due to the fact that collagen does not diffuse into PDMS surfaces of any elasticity.

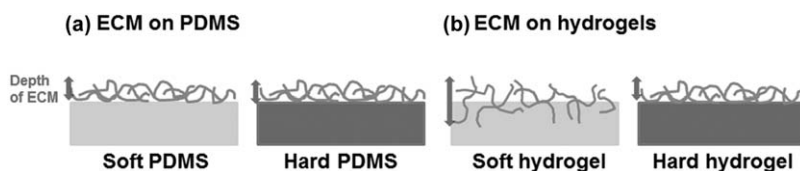


Figure 4.20 Morphologies of ECM on PDMS (a) and hydrogel (b) with hard and soft properties.¹⁷

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Rowlands and his colleagues investigated the interplay of adhesive ligand presentation and elasticity, exemplified by the finding that osteogenic induction of hMS cells generated extensively only on collagen type I-immobilized surfaces with the highest stiffness of evaluated biomaterials.¹³¹ The control of myogenic and osteogenic transcription factors by several ECMs showed that biomaterial stiffness alone did not directly guide lineage specification of stem cells. However, the combination of specific ECMs and biomaterial elasticity (*e.g.*, the elasticity of specific ECMs), directs stem cell fate of induction into specific lineages in 2D cultivation.

The induction of hPS cells into cardiomyocytes was generally reported to perform on stiff TCP plates that do not resemble the physiological tissue mechanics in both mature or developing hearts. The cultivation system that incorporates factors mimicking the mechanical microenvironment of the hearts (*i.e.*, elasticity in cell cultivation materials) should be important toward the cardiac induction of hPS cells. Then, Hazeltine and his colleagues studied the temporal influence of the stiffness of cell cultivation materials on the differentiation of hES (H9) cells into cardiomyocytes on crosslinked PAAm hydrogels with a physiologically relevant range of elasticity, having 4400, 18 400, 49 400, and 76 000 Pa of elastic moduli that were surface coated with Matrigel.⁹¹ The induction of hES cells cultivated on PAAm hydrogels into cardiomyocytes was performed using a GiWi protocol (Type E protocol in Figure 4.3) where both a Wnt inhibitor (IWP4) and a GSK3 inhibitor (CHIR99021) were used. Briefly, on day 0, hES cells were cultured in RPMI/B27 media without insulin supplementation with CHIR99021. (b) On day 1, the media was exchanged with RPMI/B27 media without insulin. On day 3, the media was exchanged with RPMI/B27 without insulin supplementation with IWP4. On day 5, the media was exchanged with RPMI/B27 without insulin. On day 7 and every 3 days following, the media was exchanged with RPMI/B27 media.⁹¹

Brachyury (T) expressed at the maximum in hydrogels having intermediate stiffness (50 000 Pa) on 24 h of the directed induction of hES cells, indicating that the elasticity of the cell cultivation hydrogel had a large impact on the initial induction trajectory of hES cells toward mesendoderm. On day 15 of induction, the cardiac troponin T (cTnT, cardiomyocyte marker) in hES cells also peaked on the hydrogels having intermediate elasticity (50 000 Pa) (Figure 4.21A and B).⁹¹

Human ES cells were initially induced toward cardiomyocyte differentiation on TCP plates for 6 days at an Nkx2.5/Is1⁺ cardiac progenitor cell stage, and the cells were shifted to the hydrogel to evaluate the impact of the stiffness of cell cultivation biomaterials on the cardiomyocyte specification of mesodermal progenitors. No extensive differences in cardiomyocyte purity were evaluated from measurement of cTnT expression relative to the stiffness of the hydrogels on day 15 (Figure 4.21C).⁹¹ The results indicate that the induction of hES cells is only sensitive to stiffness of biomaterials at early stages of mesodermal induction. The optimal choice of cell cultivation materials having adequate timing and stiffness can enhance the purity of hPS cell induction into cardiomyocytes.

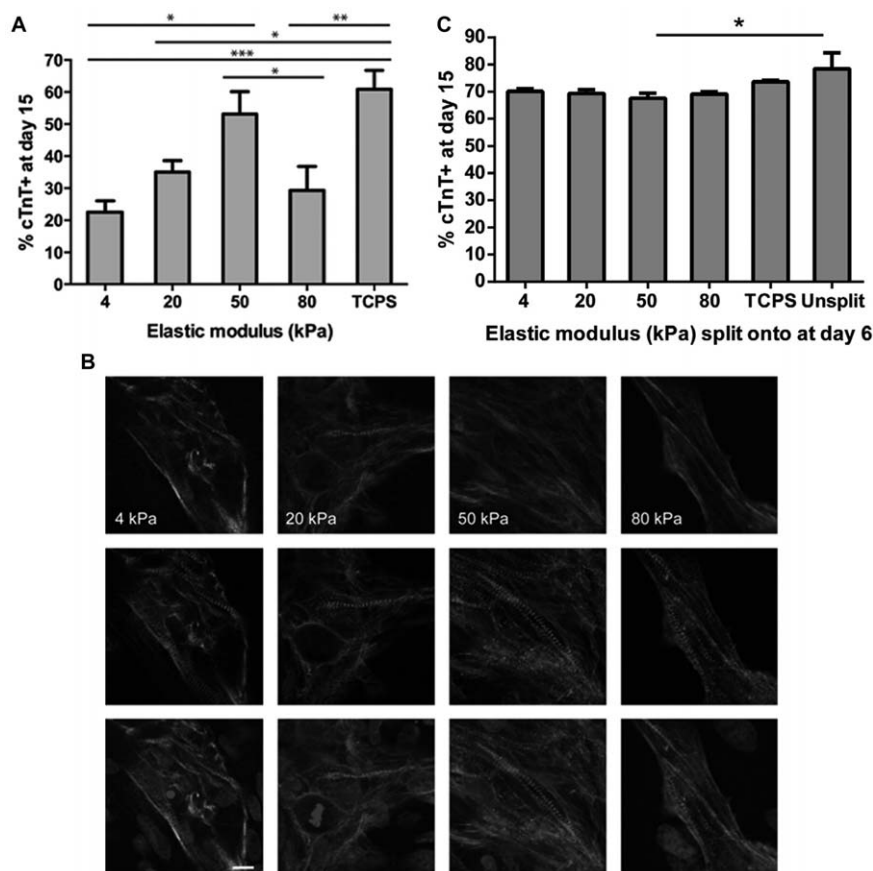


Figure 4.21 Cardiomyocyte induction on TCP dishes and polyacrylamide hydrogels of varied levels of elasticity during directed cardiomyocyte induction. (A) hES cells (H9) were inoculated onto hydrogels or TCP dishes, subjected to directed induction and fixed on day 15. cTnT expression on a 50 kPa hydrogel was extensively higher than cTnT expression on 4 and 80 kPa hydrogels. (B) Representative pictures showing organized sarcomeres in H9-derived cardiomyocytes on hydrogels of indicated stiffness on day 15. Top column = cardiac troponin I, middle column = α -actinin, and bottom column = merged pictures with staining of nuclei using Hoechst 33342. Scale bar = 10 μ m. (C) cTnT expression of hES cells on day 15, which were initially directed toward cardiomyocyte induction on TCP dishes for 6 days at a Nkx2.5/Is1⁺ cardiac progenitor stage and then shifted to hydrogels having different stiffness.⁹¹ Adapted from ref. 91 with permission from Elsevier, Copyright 2014.

It is summarized that Engler's landmark work suggesting that cell culture matrices guide stem cell fate of induction fate is only valid under the restricted condition that the stem cells are cultivated on hydrogel with immobilization of collagen type I in a 2D system during early stage of differentiation where ECMs (*e.g.*, collagen type I) are able to penetrate the

hydrogel surface to some extent, and not on rigid biomaterials where ECMs are not able to penetrate the material surface, such as metal, glass, or PDMS. Hydrogel having different elasticity leads to heterogeneity of ECM anchoring densities, subsequently changing the mechanical feedback of ECMs on stem cell. When the ECM is much loosely bound on soft hydrogel, ECM does not generate the mechanical feedback where the integrin complex needs to make clusters in FA and to generate signals *via* ERK/MAPK. The mechanical feedback in stem cell guide stem cells into adequate induction lineages or leads stem cells to stay at a pluripotent state (undifferentiated state).

4.3.1.6 Contradictory to Engler's Research in 3D Cultivation

Stem cells can dictate and react to mechanical properties of the ECMs as discussed in a previous section. However, it is difficult to evaluate how ECM mechanics guide stem cell fate in 3D microenvironments biophysically. Huebsch and his colleagues discussed how the lineage commitment of MS cells varied in response to the stiffness of 3D microenvironment. The highest osteogenic differentiation of MS cells was extensively observed in alginate gel immobilized with RGD peptide (RGD-grafted alginate) at 11 000–30 000 Pa, whereas MS cells predominantly induced differentiation into the adipogenic lineage in soft alginate gels (2500–5000 Pa).¹⁵⁶ The tendency for material elasticity to give influence to induction lineages of MS cells was also recognized in several kind of hydrogels, for example, RGD-grafted PEG hydrogels and RGD-grafted agarose.¹⁵⁶ The relationship between differentiation direction and stem cell morphology in 3D hydrogel was studied to investigate the effect of hydrogel elasticity on stem cell morphologies in 3D cultivation that correlate with differentiation fate of MS cells. Unfortunately, the elastic modulus did not give any significant influence on morphologies of MS cells in the 3D hydrogel.¹⁵⁶ This result indicates that stem cell fate of differentiation did not have correlation with cell morphologies in the 3D hydrogel, in contrast to previous work in 2D studies.¹⁶ However, biomaterial elasticity controlled the nanoscale reorganization of adhesive ligand and integrin binding, both of which correlated with osteogenic commitment of MS cells and were traction-dependent.¹⁵⁶ We found that the stem cell used traction force to sense the RGD oligopeptides displayed by the hydrogels on a nanometer scale, clustering RGD oligopeptides near integrin, whereas the oligopeptides remained to attach on the hydrogels (Figure 4.22).¹⁵⁶ RGD clustering became to be maximized in hydrogel having intermediate stiffness (22 000 Pa). This result is elucidated from the fact that stem cell in extremely soft hydrogel is difficult to coagulate the cytoskeleton-associated adhesive complexes, which are required to exert traction force, maintain the RGD-integrin complex, and prevent them from deformation of the hydrogel to create RGD clustering.¹⁵⁶ Blocking RGD-integrin binding to α_v and α_5 integrins by using anti- α_v - or anti- α_5 -antibodies extensively increased adipogenic differentiation and reduced osteogenic differentiation in RGD-grafted alginates in 3D cultivation in an antibody dose-dependent fashion.

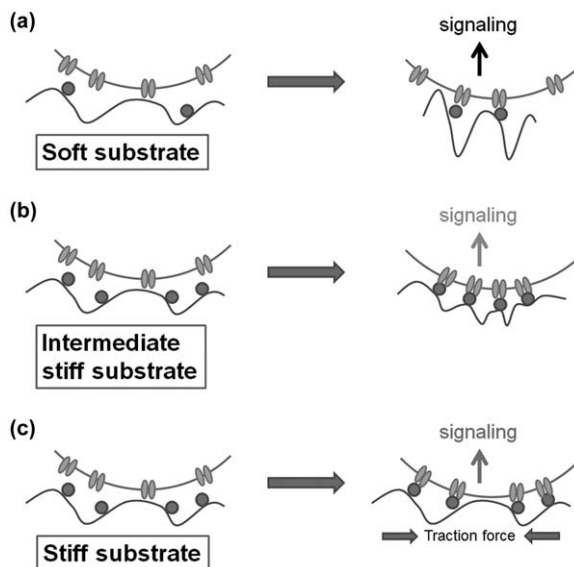


Figure 4.22 Matrix stiffness (stiff substrate [c], intermediate substrate [b], and soft substrate [a]) controls reorganization of adhesion ligands and integrin binding, which are traction-dependent, at the nanometer levels.¹⁷ Reproduced from ref. 17 with permission from American Chemical Society, Copyright 2013.

On the other hand, only α_5 integrin showed expression on the surfaces of MS cells in 2D cultivation and were used to induce into osteoblasts.¹⁵⁶

MS cells can sense difference in the physical characteristics of adhesive biomaterials as changes in adhesive ligand presentation. MS cells can be used as sensors to mechanically process biomaterials into structures that feed back to control their fate.

4.3.2 Topographic Effects of Biomaterials on the Differentiation Fates of hPS Cells

Topographic surface at nano- or microscale level regulates induction fate of stem cells. This is because cell function is controlled by complicated topographical niche *in vivo*, such as ECM geometries. The nano- and microscale properties of topographic surface promote changes in gene expression, proliferation, migration, elongation, polarization, and cell alignment.^{39,194–196} MacBeath and his colleagues investigated how topographic properties gave influence on the differentiation fate of MS cells. They found that MS cells allowed to spread on large island extensively differentiated into osteoblasts, whereas MS cells that were restricted from spreading on small island of ECMs predominantly differentiated into adipocytes.¹⁵² On the other hand, the effect of mechanical environment on hPS cells was less sufficiently evaluated. Table 4.6 summarizes examples of the topographic effects of materials on

Table 4.6 The effect of topography (physical factor) of materials on hPS cell induction.¹⁸ Adapted from ref. 18 with permission from the Royal Society of Chemistry.^a

hPSCs	Cell culture materials	Physical effect (type)	Cell type (%)	Ref. (year)
<i>Topography of biomaterials</i>				
hESC (H9)	Matrigel-coated polyurethane dishes	Topography (size of EB) (Type B)	Cardiomyocytes	27 (2010)
hESC	Agarose hydrogels (2D or 3D)	Topography (size of EB) (Type AB)	Cardiomyocytes	25 (2013)
ESC-CM from hESCs (H9)	Matrigel-coated plates	Topography (micropattern) (Type G and Type H)	Cardiomyocytes (cTnT 98%)	104 (2014)
hESCs, hiPSCs	Gelatin-patterned polyacrylamide gels	Topography (micropattern) (Type A)	Cardiomyocytes	33 (2015)
hESCs (H9)	Gelatin-coated polyurethane acrylate ridge/groove pattern array	Topography (micropattern) (Type E)	Neuron	85 (2010)
hESCs (HES-3, H7), hiPSCs (iPS-IMR90)	Matrigel- and laminin-immobilized TCPS	Topography (micropattern) (Type B and Type E)	Neural cells	48 (2013)
hiPSCs (SFS.1)	Nanograting PDMS	Topography (micropattern) (Type A and E)	Neuron	39 (2013)
hESCs (H1)	PLO/LN coated nanograting PDMS	Topography (micropattern) (Type E)	Neuron	110 (2015)
hESCs (H1)	Microchannel PLGA coated with Matrigel	Topography (micropattern) (Type B)	Retinal cells	55 (2012)
<i>Topography of biomaterials (nanofibers)</i>				
hESCs [WA01 (H1), WA09 (H9)]	PDL or Matrigel coated pDTEC electrospinning nanofibers	Topography (nanofibers) (Type E)	Three germ layer cells	82 (2012)
hESCs (BG01V)	Chitosan/PCL electrospinning nanofibers	Topography (nanofibers) (Type E)	Three germ layer cells	83 (2012)
hiPSCs	PCL electrospun nanofibers	Topography (nanofibers) (Type B)	Definitive endoderm cells	40 (2014)

Table 4.6 (Continued)

hPSCs	Cell culture materials	Physical effect (type)	Cell type (%)	Ref. (year)
hESCs (Royan H5)	Matrigel-coated polyamide (Ultra-Web) electrospun nanofibers	Topography (nanofibers) (Type E)	Hepatocytes	84 (2010)
hESCs (KhES3), hiPSCs (Toe, 201B7)	Polyamide electrospun nanofibers (Ultra-Web) coated with Matrigel	Topography (nanofibers) (Type E)	Hepatocytes	106 (2013)
hESCs (BG01)	PLLA nanofibers (phase separation)	Topography (nanofibers) (Type B)	Osteoblasts	37 (2010)
hiPSCs	Plasma-treated PES electrospun nanofibers	Topography (nanofibers) (Type B)	Osteoblasts	43 (2013)
hESCs (BG01v)	Chitosan/PCL electrospun nanofibers	Topography (nanofibers) (Type E)	Myocytes (MHC 63%, MyD 22%)	87 (2013)
hESCs (H7)	Self-assembled nanofiber hydrogel coated with HyA	Topography (nanofibers) (Type F)	Cardiomyocytes	120 (2013)
hESC (SA002)	Polyether-based polyurethane electrospun nanofibers	Topography (nanofibers) (Type E)	Neuron	86 (2009)
hESCs (H9)	LN and PLO-coated dishes and heparin-grafted PLLA electrospinning nanofibers	Topography (nanofibers) (Type B and Type D)	Neuron	49 (2010)
hESCs (Royan H6)	Matrigel-coated Ultra-Web (polyamide) electrospun nanofibers	Topography (nanofibers) (Type E)	Neuron (β III-tubulin+ cells, 82%), (MAP2+ cells, 66%)	98 (2011)
hESCs (SA121)	Polyurethane electrospun nanofibers coated with PLO and LN	Topography (nanofibers) (Type E)	Neuron	88 (2014)

^aCOL, collagen; FN, fibronectin; LN, laminin; PLO, poly-L-ornithine; PLLA, poly-L-lactide.

hPS cell differentiation.^{25,27,33,37,39,40,43,48,49,55,82–88,98,104,106,110,120,130} In this session, we examine the topographic effect of materials on the differentiation fate of hPS cells.

4.3.2.1 Preparation of Nano- and Micropatterned Materials

Nano- and micropatterned surfaces are the most typical materials used to evaluate the effect of cell cultivation topographies on stem cell induction. Photolithography patterning (hard lithography) and microcontact printing (soft lithography) protocols are commonly used to make nano- or micropatterned material surface. Several microdomains, sizes, morphologies, and shapes of stem cell adhesive models can be created using both of these protocols.

Several examples of the morphologies and/or shapes of nano- and microdomains of patterned surface are displayed in Figure 4.23. Patterned micropost (cylinder) (Figure 4.23[g])¹⁹⁷ and cave (Figure 4.23[h])^{198–200} structure has also been proposed. Microgrooved and striped surface morphologies have been designed for stem cell cultivation (Figure 4.23[f]).²⁰¹ Surface patterning of squares conjugated with narrow lines have also been designed (Figure 4.23[e]).²⁰² Grid lines, striped lines, stars, squares, and circles, are generally used as stem cell adhesive macro-domains (Figure 4.23[a, b, c, d]).^{203–205}

A typical manufacturing process of microcontact printing for the 2D cultivation of stem cells is described in Figure 4.24. At first, Elastomeric stamp made of PDMS is created using molds. The molds can be prepared by spin-coating a photoresist solution onto glass cover plates or silicon wafers and

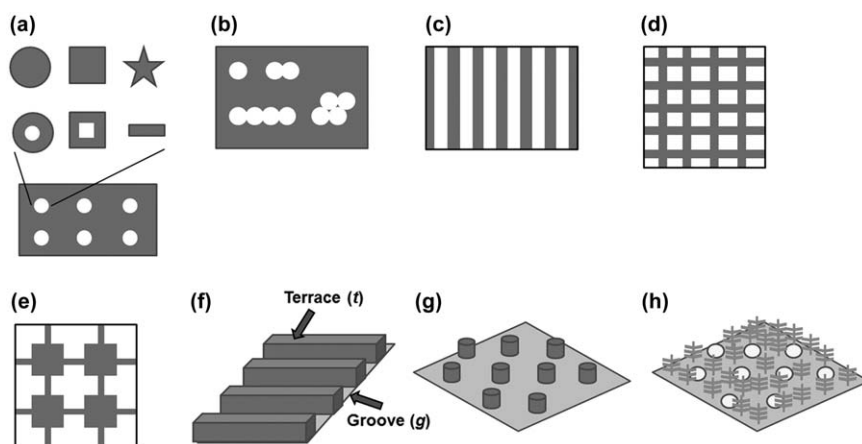


Figure 4.23 Illustration of morphologies and shapes of micropatterned biomaterials examined in the literature. Stars, squares, and circles (a), circle combinations (b), stripes (c), grids (d), grid and square combinations (e), microgroove morphologies (f), microposts (g), and circles surrounded by polymer brushes (h) are described.¹⁷ Reproduced from ref. 17 with permission from American Chemical Society, Copyright 2013.

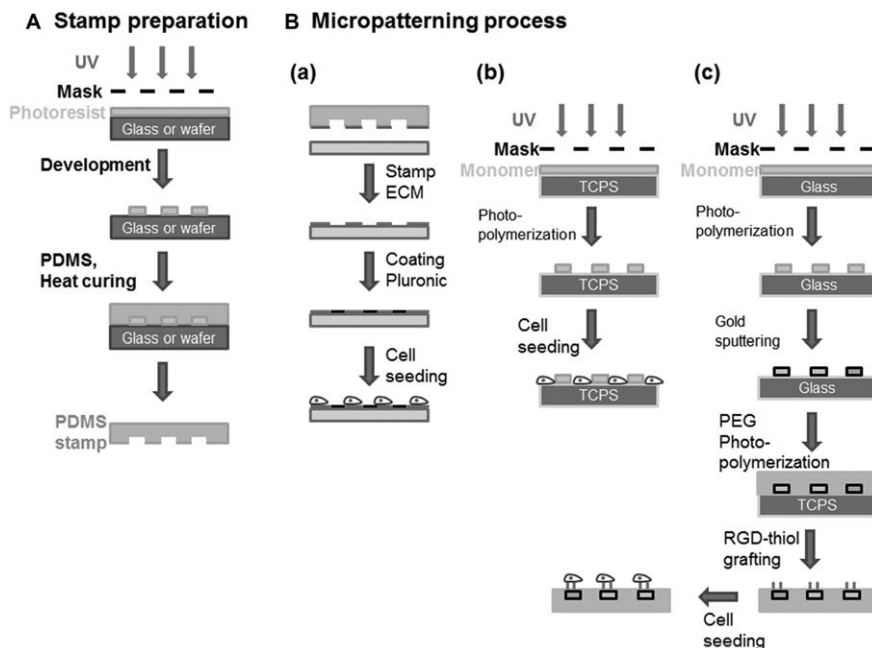


Figure 4.24 Typical microcontact printing preparation processes in 2D stem cell cultivation. Stamp preparation method (A) and several micropatterning processes (B).¹⁷ Reproduced from ref. 17 with permission from American Chemical Society, Copyright 2013.

subsequently exposure of the photoresist to UV (ultraviolet) light *via* a mask having the geometric features that were designed (Figure 4.24A).²⁰³ Uncrosslinked photoresist (polymer) is detached by washing the glass cover plate or silicon wafer with solvent. The curing agent pre-polymers and PDMS are injected into the resulting molds and cured at an elevated temperature (around 70 °C) for 7 h before the PDMS stamps are detached from the molds.²⁰³ ECM solution [e.g., LN, collagen type I, Matrigel,^{206–208} fibronectin,^{152,203,209} or PLL (poly-L-lysine) solution²⁰²] are stamped on glass slides, TCP plates or slides,^{206–208} Petri dish.²⁰² or PDMS plate.^{152,209} Because stem cells extensively attach to PLL or ECM domain, non-specific adhesion outside the microcontact patterning surfaces on glass slides, TCP plates, or PDMS plates would be reduced. Because stem cells attach to the material surface through proteins, such as a triblock copolymer of PEO (polyethylene oxide)-PPO (polypropylene oxide)-PEO, Poloxamer or Pluronic,^{210–212} are commonly pasted on micropatterned slides or plates after stamping with PLL or ECM. Furthermore, PLL or ECM is stamped on the plates pasted with plasma-polymerized PEO using microstamps of PDMS where PEO is one of the low-protein-binding polymeric materials.²⁰² Microprinting is an easy protocol to create a micropatterned surface. It should be noted that the drawback of

microprinting PLL or ECM is the low stability of the remaining micropatterned PLL and ECM on the material surface because of their physical adhesion.

SAM (self-assembly monolayer) protocol can be merged with microcontact printing method. Wan and his colleagues developed micropatterned FN (fibronectin) using the SAM protocol.²⁰³ An adherent SAM octadecanethiol was shifted using PDMS microstamp onto a gold-coated glass slide. Then, the non-stamped area of the slide was coated with non-adhesive EG (ethylene glycol)-conjugated SAM. Then, the patterned surface was coated with FN solution, because FN extensively absorbed on the hydrophobic region of SAM octadecanethiol in the microcontacted area.²⁰³

Hydrogel such as PVA (polyvinyl alcohol) and PEO (PEG) have characteristics of low proteins-binding property. Chen and his colleagues used a simple and interesting micropatterning protocol (Figure 4.24B[b]).^{198,200} They prepared photo-crosslinkable azidophenyl-conjugated PVA.^{198,200} The photo-crosslinkable PVA solution was located on TCP plates and crosslinked through micropatterned photomasks. The PVA was crosslinked and used to make hydrogel on the outside of stem cell cultivation sites. This led stem cells to be cultivated on micropatterned TCP plates surrounded by cross-linked hydrogel. If necessary, ECM are coated on the micropatterned TCP plates. A similar strategy, but using another protocol, was investigated by Connelly *et al.*¹⁹⁹ Micropatterned stamp inked with ω -mercaptoundecyl bromoisobutyrate (thiol initiator) was used into conformal contact with a gold-coated coverslip to locate the initiator as self-assembled monolayers.¹⁹⁹ ATRP (atom transfer radical polymerization) of OEGMA [oligo(ethylene glycol) methacrylate] was used on the gold-coated coverslip. Then, the micropatterned coverslip was coated with collagen type I that was attached on the sites with no micropatterned OEGMA polymer brushes. Stem cells were cultivated on bowls surrounded by polymeric hydrogels on micropatterned plates made from either OEGMA polymer brushes or PVA, letting it possible to reduce the stem cells from leaving the bowl where stem cells were intended to locate (Figure 4.23[h]).

Tang and his colleagues investigated a little different preparation protocol for the micropatterned surface discussed above (Figure 4.24B[c]).²⁰⁴ Clean glass plate was spin-coated with positive photoresists, exposed to UV light *via* masks with the designed geometric feature, and developed. Then, the surfaces were sputtered with gold, and the non-polymerized photoresists were removed. Allyl mercaptan was immobilized on the gold microislands *in vacuo*. These procedures were important to shift the gold onto the surfaces of the PEG hydrogels in the following process. PEG diacrylate with photo-initiators was casted onto the micropatterned glass and photo-crosslinked under irradiation of UV. Micropatterned PEG hydrogels were subsequently generated by detaching the hydrogels bound with the gold micropatterns from the glass plates. Cyclo(RGDfk)-thiol (k: lysine; f: D-phenylalanine; D: aspartic acid; G: glycine; R: arginine) was conjugated onto the gold microisland on the PEG hydrogel. Stem cells could adhere on the RGD microdomain of the PEG hydrogel through the integrin receptor.

Microgrooved surface with striped groove and terrace (Figure 4.23[f]) could be developed using photolithographic methods. Beduer and his colleagues developed microgrooved surface on PDMS, and LN and PLL were coated on the microgrooved surfaces for NS cell cultivation.²⁰¹

4.3.2.2 Uniform EB Generation in Micropatterned Surface

The size of EBs is an essential point, which affects the induction of hPS cells into several cell lineages as is described in Section 4.2.1. Then, the use of micropatterned surfaces to form homogeneously sized EBs generates more homogenous hPS cell induction into several cell lineages than does the formation of EBs in non-adherent cell cultivation plates in suspension, such as in Types A to D and Type AB induction methods.^{25,27}

4.3.2.3 Osteogenic and Adipogenic Induction of Stem Cells on Micropatterned Biomaterials

It has been demonstrated in 2D cultivation that cell shape control by micropatterned biomaterials should lead to the stem cell commitment into several lineages. Some investigators have demonstrated the effect of the shape and spreading area of stem cells cultivated on micropatterned biomaterials on induction lineage of the commitment.^{152,200,201,203,205,213–218}

Table 4.7 shows several researches of stem cell induction into osteoblasts and adipocytes on micropatterned biomaterials.^{152,198,200,203,204,214–217,219}

We know that cell inoculation density directly give influence on hMS cell lineage commitment; hMS cells at low inoculation density tend to induce into osteoblasts, and hMS cells at high inoculation density like to induce into adipocytes when cultivated in mixed differentiation media for adipocyte and osteoblast (Figure 4.25[d]).¹⁵² These results are elucidated by (a) an enhancement in cell–cell contact and paracrine signaling and/or (b) a reduction in spreading and cell adhesion on the cell cultivation biomaterials. On the other hand, typical TCP cell plates cannot distinguish between these two potential effects. Therefore, McBeath and his colleagues studied the effect of cell shapes on the commitment of stem cell induction by regulating the degree of cell spreading in the absence of cell–cell communication.¹⁵² They placed square-shaped microcontact fibronectin prints onto PDMS plates to form “islands” of FN surrounded by area coated with Pluronic F108, which makes stem cells to attach on FN domains (Figure 4.23[a]).¹⁵² Human MS cells were inoculated on the micropatterned PDMS surface by adhesion as single cells per island and spread to varying degrees, which depends on the island size (10 000 and 1000 μm^2) in mixed induction media for osteogenic and adipogenic differentiation of hMS cells. Adipogenic differentiation was found only on small island, suggesting that round hMS cell morphologies led to the induction commitment of hMS cells into adipocytes. On the other hand, osteogenesis was found only on the large domain of islands, indicating that hMS cell spreading promotes osteogenic

Table 4.7 Some investigations into stem cell induction on micropatterned material surfaces (adipogenic and osteogenic induction).¹⁷ Adapted from ref. 17 with permission from American Chemical Society, Copyright 2013.^a

Stem cell source	Micropatterned materials for stem cell culture	Pattern type	Medium	Differentiation	Ref. (year)
hMSCs	Micropatterned amorphous diamond, titanium, tantalum, and chromium with square shape on silicon wafer	Figure 4.23[a]	Differentiation medium	Osteoblasts	214 (2010)
Murine MSCs	Micropatterned PDMS with grid (lattice) morphology coated with fibronectin	Figure 4.23[d]	Differentiation medium	Osteoblasts	219 (2011)
hADSCs	Micropatterned fibronectin with ring shape or rectangles on gold-coated slides	Figure 4.23[a]	Differentiation medium	Osteoblasts, adipocytes	203 (2010)
hMSCs	Micropatterned fibronectin with square, rectangular, flower, and star shape on octadecanethiol surface	Figure 4.23[a]	Mixed differentiation media of adipocytes and osteoblasts	Adipocytes, osteoblasts	215 (2010)
hMSCs	Micropatterned fibronectin with square shape on PDMS surrounded by Pluronic F108	Figure 4.23[a]	Mixed or single differentiation medium of adipocytes and osteoblasts	Adipocytes, osteoblasts	152 (2004)
Rat MSCs	Micropatterned RGD with circle, square, triangle, and star shape on PEG hydrogel	Figure 4.23[a]	Differentiation medium	Adipocytes, osteoblasts	216 (2011)
hMSCs	Micropatterned alkane thiol surface with circle, octagon, triangle, trapezoid, square, and pentagon shape surrounded by PEG-terminated alkanethiol on gold surface	Figure 4.23[a]	Differentiation medium	Adipocytes	198 (2008)
Rat MSCs	Micropatterned RGD with circle and aggregated circle shape on PEG hydrogel	Figure 4.23[b]	Differentiation medium	Adipocytes, osteoblasts	204 (2010)
hMSCs	TCPS surface of circle shape surrounded by micropatterned polyvinyl alcohol	Figure 4.23[h]	Differentiation medium	Adipocytes, osteoblasts	200 (2011)
hMSCs	TCPS surface of triangle, square, pentagon, hexagon, and circle shape surrounded by micropatterned polyvinyl alcohol	Figure 4.23[h]	Differentiation medium	Adipocytes	217 (2011)

^ahADSCs, human adipose-derived stem cells; hMSCs, human MSCs; MSCs, mesenchymal stem cells; PDMS, polydimethylsiloxane; TCPS, tissue culture polystyrene.

induction (Figure 4.25[a]). This evidence shows that the control of cell shape alone is able to regulate a switch in hMS cell commitment between osteogenic or adipogenic fates in 2D cultivation.¹⁵² Shape-mediated commitment was promoted by actin-cytoskeleton expression. This is because the disruption of the actin cytoskeleton by the injection of the Rho kinase inhibitor (ROCK) Y-27632 or the actin-disrupting agent cytochalasin D into the mixed induction media reduced osteogenesis and enhanced adipogenesis of hMS cells in the cases of inhibitor addition where the inhibitor was included in myosin activation. The commitment change between osteoblast or adipocyte

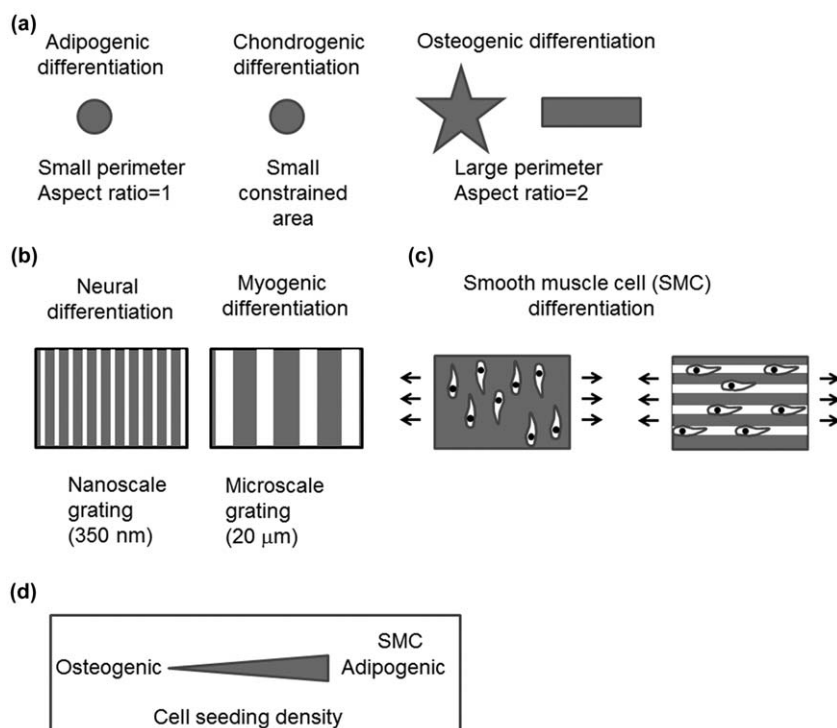


Figure 4.25 Stem cell induction on some different micropatterned biomaterials. Osteogenic stem cell induction is facilitated on biomaterials with relatively high aspect ratios and larger perimeters. Chondrogenic stem cell induction is facilitated on biomaterials of constrained area. Adipogenic stem cell induction is facilitated on biomaterials with lower aspect ratios and smaller perimeters (a). Biomaterials with microscale grating facilitate myogenic stem cell differentiation, whereas biomaterials with nanoscale grating facilitate NS cell differentiation (b). Micropatterned biomaterials with striped groove (grating) morphologies facilitate stem cell induction into smooth muscle under uniaxial strain (c). Higher cell inoculation density facilitates the induction of stem cells into smooth muscle cells and/or adipocytes, whereas lower cell inoculation density facilitates osteogenic stem cell differentiation (d).¹⁷ Reproduced from ref. 17 with permission from American Chemical Society, Copyright 2013.

seemed to be mediated *via* the ROCK-RhoA signaling pathway; RhoA activity and cell shape control were both important, but neither was sufficient to regulate the change in the commitment of hMS cells.¹⁵²

Cell morphology is able to be controlled by the shape of the adhesive area on the micropatterned biomaterial. Then, Kilian and his colleagues extensively studied the effect of subcellular curvature, aspect ratio, and adhesion area of micropatterned biomaterials on hMS cell commitment into osteoblasts and adipocytes. Human MS cells were cultivated on micropatterned FN domains with flower, star, and rectangular shapes on glass dishes at changing curvatures and aspect ratios in mixed induction media for osteoblast and adipocyte. The aspect ratio was calculated as the ratio of the length of FN shape at its long axis to its length at its short axis (Figure 4.23[a]).²¹⁵ Human MS cells were induced into osteoblasts when they were cultivated on large domain (*e.g.*, 5000 μm^2). In contrast, most of the cells were induced into adipocytes in the mixed induction media, when hMS cells were cultivated on small domain (*e.g.*, 1000 μm^2) of different shapes (Figure 4.25[a]). These results suggest that the size of the cell adhesive area extensively regulates induction fate of stem cells, as reported in McBeath and his colleagues.¹⁵² Human MS cells cultivated on some patterns of intermediate area (2500 μm^2) were induced into a mixed lineage of osteoblast and adipocyte. Then, the effect of curvature, aspect ratio, and shape of the cell adhesive area on hMS cell induction commitment was evaluated at a constant cell adhesive area of 2500 μm^2 in mixed induction medium.²¹⁵

Human MS cells cultivated on rectangular domain with aspect ratios of 4:1, 3:2, and 1:1 showed that osteogenic differentiation enhanced with increase of aspect ratio (Figure 4.25[a]). Human MS cells cultivated in rectangle domain with aspect ratios of 4:1 were 60% osteogenic induction, whereas hMS cells cultivated in square domain (aspect ratio 1:1) were only 45% osteogenic induction.²¹⁵ Human MS cells cultivated on star-shaped domain with sharp points and concave edges at the vertices expressed 60% osteogenic differentiation, whereas hMS cell culture on flower-shaped domain with large convex curve along each edge gave 60% adipogenic induction. Human MS cells cultivated on pentagon-shaped domain with straight line for the edge was induced into both osteoblast and adipocyte.^{198,215,216} This information is valuable in that the subtle geometric change of stem cell adhesive sites are extensively key factors in guiding lineage commitment of hMS cell differentiation in 2D cultivation. It was demonstrated that the shape of stem cells solely can affect the direction of their induction in 2D cultivation.^{198,215} Stem cell shape is guided by cytoskeleton factors, such as FA complexes and stress fibers. Human MS cells cultivated on the flower shape domain displayed less stress fiber and FA than those cultivated in star-shaped domain. Moreover, a less degree of actomyosin contractility along the edges was found in immunostaining image of myosin IIa in hMS cells cultivated in the flower shape domain compared to that in star-shaped domain.²¹⁵ It is because local curvatures of stem cell shape that contractile stem cell cytoskeleton and enhance

cytoskeletal tension facilitate osteogenic differentiation relative to adipogenic differentiation. Studies of pathway inhibition and microarray assay indicate that stem cell contractility facilitate osteogenic differentiation by increasing ERK1/2 (extracellular-related kinase) and JNK (c-Jun N-terminal kinase) activation in conjunction with enhanced Wnt (wingless-type) signal (downstream effector of ROCK and RhoA signal).²¹⁵ The geometric shape of stem cell cultivated on different adhesive domains plays key points in orchestrating paracrine/autocrine factors and mechano-chemical signals, which can guide hMS cells to optimal lineages of induction fates.

Chen and his colleagues demonstrated whether differing geometries with small surface domain (approximately $1000\ \mu\text{m}^2$) have an influence on the adipogenic differentiation of hMS cells in an adipogenic differentiation media.²¹⁷ In this research, micropatterned TCP plates coated with photo-crosslinked PVA with cell adhesive domain of circles, hexagons, pentagons, squares, and triangles of constant surface area was used in a single hMS cell cultivation. The cellular morphologies of the hMS cells were selected as approximately the same geometries of the micropattern domain. Human MS cells cultivated on domains with different micropatterns extensively assembled actin filaments along the peripheral edge of the micropatterns, which indicates that the cells were dictating their peripheral micro-environment.²¹⁷ On the other hand, hMS cells cultivated on non-patterned TCP plates had much higher stress fibers and actin filaments in their central and peripheral regions. Human MS cells cultivated on islands of different geometries with small surface area indicated similar adipogenic induction abilities.²¹⁷ However, hMS cells cultivated on islands of micropatterned geometries induced into adipogenesis at extensively higher degree than hMS cells cultivated on non-patterned TCP plates, which was consistent with the studies reported by some investigators that smaller spreading areas prefer adipogenic induction of hMS cells.^{152,215}

Wan and his colleagues demonstrated the induction and proliferation abilities of hADS (human adipose-derived stem) cells on several size and shape of micropatterned biomaterials on which hADS cells were cultivated.²⁰³ The micropatterned biomaterials had ring (200 or 100 μm in width and 1000 or 500 μm in diameter) or rectangular structure (200 or 100 μm wide and 1000 or 500 μm long) coated with FN on microprinted SAM octadecanethiol on a gold-coated glass plates.²⁰³ The highest rates of cell expansion were found along the short axes of the rectangle pattern and at the outer edge of the ring pattern, whereas hADS cell morphologies were spreading and big.²⁰³ Human adipose-derived stem (ADS) cells, which displayed high osteogenic and adipogenic induction enhanced on region next to the narrow end of rectangle and on the inner edge of the ring structure, whereas hADS cell morphologies were elongated and small. These observations can be explained from the cytoskeletal tension originated from cell shapes.²⁰³

Micropatterned biomaterials make it possible to investigate the effect of stem cell shape and spreading on single-cell induction by the cultivation of a single cell on several patterned domains; we can also investigate the effect of

cell-cell contact between stem cells on cell induction by regulating cell contact number through the design of adequate micropatterned biomaterials. Tang and his colleagues investigated some interesting micropatterned apase conjugated with RGD on PEG hydrogel as illustrated in Figure 4.26.²⁰⁴ The micropatterned space was consisted of 1–9 microislands of 30 μm in diameter, which allow single cells to attach on each micropatterned circle. The coordination numbers per island were calculated to be 3.6, 2.5, 1.5, 1, and 0 in microdomain consisting of 9, 4, 4, 2, and 1 microislands, respectively, as demonstrated in Figure 4.26.²⁰⁴ The investigators recognized that an early marker of osteogenesis, ALP, enhanced with the increase of coordination number. Adipogenic induction of MS cells, as evaluated by Oil Red O staining (counting cells with lipid droplet), also enhanced with the increase of coordination numbers.²⁰⁴ Inhibiting gap junction between cells with AGA (18 α -glycyrrhetic acid) treatment extensively restricted osteogenic and adipogenic induction of MS cells on all microdomains, which did not depend on coordination number.²⁰⁴ The micropatterned surface makes it possible to study the effect of gap junction and cell-cell contact on induction potential of stem cells.

The effect of topographical variation on osteogenic induction of murine MS cells has also noticed. Seo and his colleagues investigated micropatterned

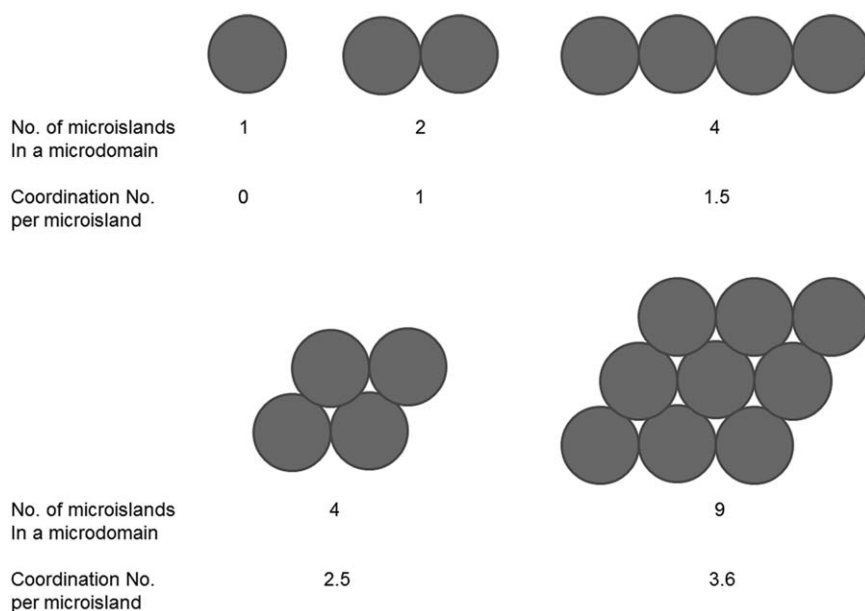


Figure 4.26 Micropatterned domains composing of 1–9 microislands with 30 μm diameters. Coordination numbers per island are evaluated to be 3.6, 2.5, 1.5, 1.0, and 0 in microdomains consisting of 9, 4, 4, 2, and 1 microislands, respectively.¹⁷ Reproduced from ref. 17 with permission from American Chemical Society, Copyright 2013.

PDMS with an ordered morphology of lattice, which consists of a constant pattern width of 2 μm and height of 1 μm , and different pattern interval of 8, 6, 4, 3, 2, 1, and 0 μm .²¹⁹

The ALP (an early marker of osteogenesis), osteocalcin (an osteogenesis marker), and gene expression of collagen type I (the major extracellular component of bone) was observed to enhance with enhanced pattern interval up until an interval of 3 μm , when murine MS cells were cultivated on the micropatterned PDMS materials coated with FN.²¹⁹ On the other hand, ALP and the gene expression of collagen type I and osteocalcin reduced with enhancing pattern interval with pattern intervals higher than 3 μm . Then, gene expression showing osteogenic induction of MS cells was the greatest on PDMS materials with pattern intervals of 3 μm on lattice micropattern morphology.²¹⁹ This evidence indicated that the topography of micropatterned surfaces is an extensively positive controller of osteogenic induction of stem cells in 2D cultivation.

4.3.2.4 Hepatic, Myogenic, and Chondrogenic Induction of Stem Cells on Micropatterned Biomaterials

Table 4.8 lists a number of investigations into stem cell induction into neural cells, hepatocytes, myocytes, or chondrocytes on micropatterned biomaterials.^{197,201,202,205–208,213,218,220–225} Chondrogenic induction of hMS cells are induced in pellet cultivation mimicking cellular aggregation during development of cartilage with exposure to TGF- β (transforming growth factor β). TGF- β is able to lead hMS cell differentiation into SMCs.²¹³ On the other hand, it is unknown which environmental parameters and cell cultivation can guide commitment between these two lineages (chondrocytes (cartilage) and SM cells). Gao and his colleagues examined differentiation shifting between SM cell and chondrocyte fates when hMS cells were cultivated on micropatterned square biomaterials (10 000 μm^2 or 1000 μm^2 islands) of FN on PDMS biomaterials prepared with the microprinting protocol.²¹³ Human MS cells on small micropatterned domains (1000 μm^2) that were restricted from flattening and spreading enhanced chondrogenic genes, while hMS cells with well-spread shapes on large micropatterned domains (10 000 μm^2) enhanced SM cell genes with TGF- β addition.²¹³ Human MS cells inducing SM cell fate expressed little change in RhoA but had extensively greater Rac1 activity than chondrogenic induced cells. The activation of Rac1 gene inhibited chondrogenic induction of hMS cells and was important for SM cell induction, whereas the activity of RhoA is considered to interact the shape-depending regulation of hMS cell lineage commitment to adipocyte and osteoblast.⁹⁵ Rac1 signal enhanced N-cadherin that was necessary for SM cell induction.²¹³ In this research, it was shown that hMS cell commitment to SM cell and chondrocyte lineages may be interacted by N-cadherin, Rac1, and cell shape.²¹³

Regulation of hES cell induction into several lineages with high efficiency (large mass and high purity) is currently not an easy work. One of the reasons for this is the inhomogeneous characteristics of hES cell colonies, whereas

Table 4.8 Some investigations into stem cell induction on micropatterned material surfaces (hepatocyte, epidermal cell, neural cell, smooth muscle cell, chondrocyte, and cardiomyocyte induction). Adapted from ref. 17 with permission from American Chemical Society, Copyright 2013.^a

Stem cell source	Micropatterned materials for stem cell culture	Pattern type	Medium	Differentiation	Ref. (year)
hESCs	Micropatterned matrigel of circular shape surrounded by Pluronic F-127 on TCPS	Figure 4.23[a]	Differentiation medium	Cardiomyocyte	208 (2009)
hMSCs	Micropatterned fibronectin with square shape surrounded by Pluronic F127 on PDMS film	Figure 4.23[a]	Expansion or differentiation medium	Smooth muscle cell, chondrocyte	213 (2010)
hESCs	Micropatterned matrigel of circular shape surrounded by Pluronic F-127 on TCPS	Figure 4.23[a]	Differentiation medium	Mesoderm cells, cardiomyocyte	207 (2008)
hMSCs	Microprinting fibronectin with stripe shape on PLGA	Figure 4.23[c]	Expansion medium	Cardiomyocyte	205 (2010)
hMSCs	Micropatterned PDMS with striped groove morphology coated with collagen type I	Figure 4.23[f]	Expansion medium	Smooth muscle cells	220 (2006)
iPSCs	Micropatterned PDMS with striped groove morphology coated with collagen type I	Figure 4.23[f]	Differentiation medium	Smooth muscle cells, neuronal cells	225 (2011)
mESCs	Micropost array made of PDMS coated with fibronectin	Figure 4.23[g]	No description	Cardiomyocyte	197 (2009)
NSCs	Micropatterned PLL with square shape connected with and without line on PEO film	Figure 4.23[a] and [e]	Differentiation medium	Neuronal cells	202 (2008)
hNSCs	Micropatterned PDMS with striped groove morphology coated with PLL and laminin	Figure 4.23[f]	Differentiation medium	Neuronal cells	201 (2012)
Adult rat hippocampal progenitor cells	Micropatterned polystyrene with striped groove morphology coated with PLL and/or laminin	Figure 4.23[f]	Differentiation medium	Neuronal cells	221 (2006)

Table 4.8 (Continued)

Stem cell source	Micropatterned materials for stem cell culture	Pattern type	Medium	Differentiation	Ref. (year)
hMSCs	Micropatterned amorphous carbon with striped groove morphology	Figure 4.23[f]	Differentiation medium	Neuronal cells	222 (2010)
hMSCs	Micropatterned PDMS with striped groove morphology coated with collagen type I	Figure 4.23[f]	Expansion medium	Neuronal cells	223 (2007)
Human epidermal stem cells	Micropatterned collagen-coated circle shape surrounded by grafted PEG on gold-coated coverslips	Figure 4.23[h]	Differentiation medium	Epidermal cells	218 (2011)
mESCs	Micropatterned protein (collagen type I, fibronectin, and growth factors) microarray with circle shape on glass slide	Figure 4.23[a]	Differentiation medium	Hepatocytes	224 (2010)

^aESCs, embryonic stem cells; hESCs, human ESCs; hMSCs, human MSCs; hNSCs, human NSCs; iPSCs, induced pluripotent stem cells; NSCs, neural stem cells; PEO, polyethylene oxide; TCPS, tissue culture polystyrene.

hES cell cultivation on a micropatterned biomaterial gives size-regulated aggregates of hES cells.^{207,208} Niebruegge and his colleagues developed homogeneously sized hES cell aggregate by cultivating hES cells on circular domains of Matrigel having 800 or 400 μm in diameter, which were prepared using micro-stamped protocols on TCP plates and coated with the non-protein adhesion Pluronic F127.²⁰⁸ After 2 or 3 days, the aggregates were transferred into bioreactors and cultivated in cardiac induction media under hypoxia (4–5% oxygen tension), when homogeneously sized hES cell aggregates were generated. The homogeneously sized hES cell aggregates could induce towards cardiomyocyte with high efficiency under hypoxia.²⁰⁸ Human ES cell aggregates after differentiation could start beating spontaneously, which indicated that homogeneously sized differentiated hES cell aggregates created several cardiomyocyte functions.²⁰⁸

Mechanical stimulation of blood vessel wall *in vivo* plays an extensive function in the induction of MS cells into vascular SM cell. Some research has indicated that mechanical strain increases induction of NCSCs and MS cells into vascular SM cell.^{220,225–228} On the other hand, after long stimulating process with cyclic uniaxial strain, MS cells oriented perpendicularly to the axis of strain (Figure 4.25[c]), which caused a reduction in SM cell markers after an initial up-regulation. The cellular alignment is different from cellular alignment reported in *in vivo* condition, whereas vascular SM cells order in the circumferential direction.^{220,229,230} Kurpinski and his colleagues cultivated hMS cells for 48 h on a micropatterned PDMS surface with striped grating morphologies of 3 μm depth, 10 μm groove spacing, and 10 μm terrace width to induce stem cells into parallel order with the axis of strain where collagen type I coated with uniaxial strain (1 Hz, 5%) (Figure 4.25[c]).²²⁰ Decreases in osteogenic/chondrogenic ECM markers and increase in contractile marker (*e.g.*, calponin 1) were found in hMS cells cultivated on the micropatterned PDMS surface under uniaxial strain.²²⁰ Proteins from cartilage matrices were detected to reduce extensively using uniaxial strain, which suggested that tensile stress reduced the phenotypes of compression-bearing tissues.²²⁰ The surface of micropatterned materials is able to control direction of cell alignment with uniaxial strain, therefore controlling the fate direction of stem cells, as in SM cells.^{220,225} The micropatterned biomaterials help to mimic *in vivo* microenvironmental properties of helical or circumferential SM cell alignment within blood vessel wall.

Tay and his colleagues studied the differentiation of hMS cells to the myogenic lineages when hMS cells were cultivated on PLGA thin film having micro-stamped FN in 20 μm stripe separated by non-adhesive gaps of 40 μm coated with Pluronic F127.²⁰⁵ Human MS cells cultivated on micropatterned biomaterials in proliferation media were observed to be extensively elongated with small adhesion domains of around 2000 μm^2 , while hMS cells cultivated on unpatterned biomaterials had flat morphology with high adhesion domains of around 10 000 μm^2 .²⁰⁵ Some hallmark myogenesis genes (β -MHC, cTnT, MyoD1, and GATA4) and neurogenesis (MAP2, GFAP, nestin, and NeuroD1) were enhanced in hMS cells on micropatterned biomaterials

in proliferation media, while osteogenic genes (RUNX2 and ALP) were extensively reduced or stayed at normal level. Cardiac myosin heavy chain (MHC), myogenic lineage proteins, extensively enhanced in hMS cells cultivated on micropatterned biomaterials.²⁰⁵ The distortion of enforced cell shape led to the rearrangement of the cytoskeletal networks and changed the nucleus shape, which indicated the mechanical deformation of hMS cells gave signals into biochemical responses and finally led to extensive induction towards a special lineage, such as the myocardial lineages.

The induction of hMS and hES cells into endoderm lineages, such as β cells and hepatocytes, might be beneficial for clinical treatment but needs complex and complicated processes. Stem cell stimulation with some small molecules and/or growth factors is important at adequate amount with appropriate duration and timing. Tuleuova and his colleagues reported a rather reliable protocol of inducing ES cells into the hepatic lineage by culture of mES cells on micropatterned protein arrays without and with micropatterned co-culture of human hepatic stellate cell.²²⁴

Protein microarrays were located on silane-modified glass plates using microarray systems with circular protein spots of 500 μ m diameter where the protein solution used for contact-printing included BMP4 (bone morphogenetic protein 4), bFGF (basic fibroblast growth factor), and HGF (hepatocyte growth factor), which were mixed with ECMs composed of collagen type I and FN.²²⁴ Murine ES cells were cultivated on the protein spots and showed hepatic induction. Co-culture with hepatic stellate cells (non-parenchymal liver cells) on the protein spots increased hepatic induction of mES cells in comparison with mES cell cultivation on the protein spots alone or with co-cultivation of the hepatic stellate cells without micropatterning biomaterials.²²⁴ Microarrayed protein domains on plates sort or control mES cells into hepatocyte lineages with good efficiency; hepatic induction of mES cells cultivated on the printed protein domains seemed to increase in comparison to mES cells in traditional cultivation with medium including the same growth factors in solution.²²⁴ Moreover, a high amount of soluble growth factors should be used in the traditional cultivation protocol, with daily medium changes using medium including growth factors, whereas growth factors in the protein domains were printed once at the starting of experiments, with sixty times less amount of total growth factor used than in the traditional cultivation media.²²⁴ This shows that protein microarray provides a more efficient protocol to present valuable growth factors to ES cells and leads to more economical growth factor usage. Growth factors bound to ECMs and conjugated on the biomaterials might have much stable and functional activities than soluble growth factors included in cultivation medium.^{224,231}

4.3.2.5 NS Cell Induction on Micropatterned Biomaterials

Neural cell induction in hPS cells has been especially investigated on micro/nano-grating (groove/ridge)-patterned array biomaterials.^{39,48,85} Pan and his colleagues examined the effect of micro/nano-grating biomaterials with

different widths (height: 300 nm; widths: 5 μm , 2 μm and 350 nm) on the commitment of hiPS cell induction into several lineages using the Type E and Type A methods described in Figure 4.3.³⁹ The micro/nano-grating biomaterials were made from PDMS using a mold and were prepared by a laser interference lithographical method. The micro/nano-grating PDMS biomaterials were coated with Matrigel for the cultivation of hiPS cells. The nuclei of the hiPS cells were observed to elongate and align in the direction of the micro/nano-grating biomaterials, whereas the hiPS cells themselves adhered randomly to flat surface (Figure 4.27),³⁹ which was found to be similar results reported by other investigators.^{17,85} Neuronal markers were

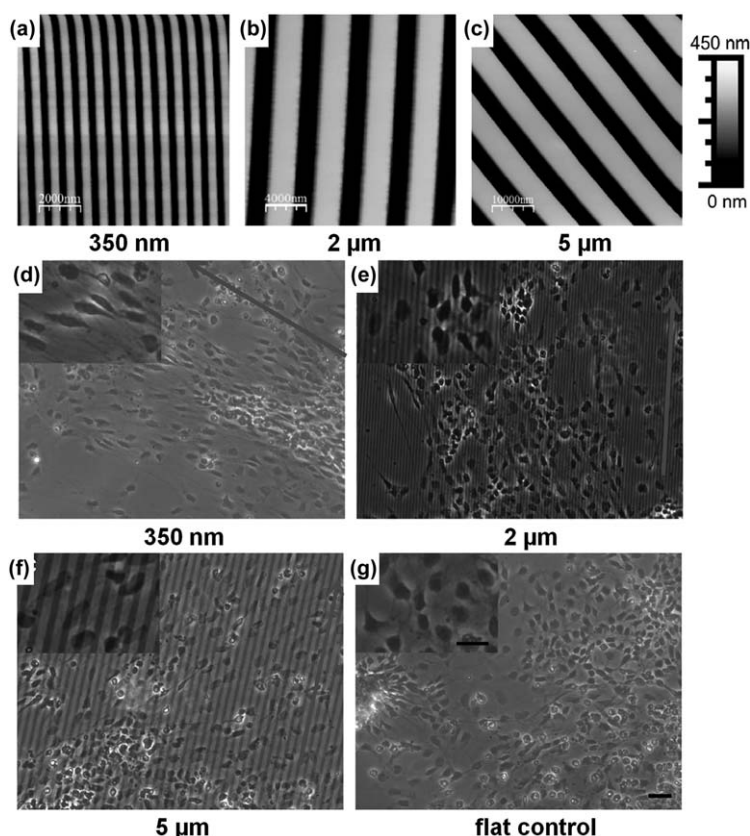


Figure 4.27 Topographic effect on hiPS cell induction towards neuronal lineage. Atomic force microscopy image of nano/microstructured PDMS surface. Groove width = ridge width = 350 nm (a), 2 μm (b) and 5 μm (c) with height = 300 nm. Morphology of hiPS cells (SFS.1) on nano/microstructured (d–f) and flat PDMS surface (g) and cell direction relative to biomaterial structure. The insertions are high magnification images of cell morphologies. Scale bar in (g) = 20 μm . Groove width = ridge width = 350 nm (d), 2 μm (e) and 5 μm (f) with height = 300 nm.³⁹ Adapted from ref. 39 with permission from Elsevier, Copyright 2013.

significantly enhanced and neurite outgrowth was found to be highly expressed on the nanograted surface either with solely topographical effects or combined with pre-neuronal induction (Figure 4.27). A width of 350 nm displayed the highest intensity of neuronal marker expression,³⁹ which was the same results investigated by Lee and his colleagues.⁸⁵ Both of this investigation³⁹ and some other research^{48,85} indicates the importance of topographical cues, specifically regarding nano-grading morphologies, in guiding the fate of hPS cell induction into neuronal cell lineages.

hPS cell-derived photoreceptors are an attractive cell source for *in vitro* models of retinal degenerative disease. However, typical characteristics of retinal cells cannot be generally developed in dissociated cell cultivation *in vitro*. Although dissociated cultivation of mammalian retinal cell commonly expresses rudimentary aspects of morphological induction, explant cultivation of mammalian retinas indicates that cells can hold extensively differentiated properties when kept in organized states.^{55,232–234} Then, it is necessary to create a protocol to develop 3D retina-like tissues from

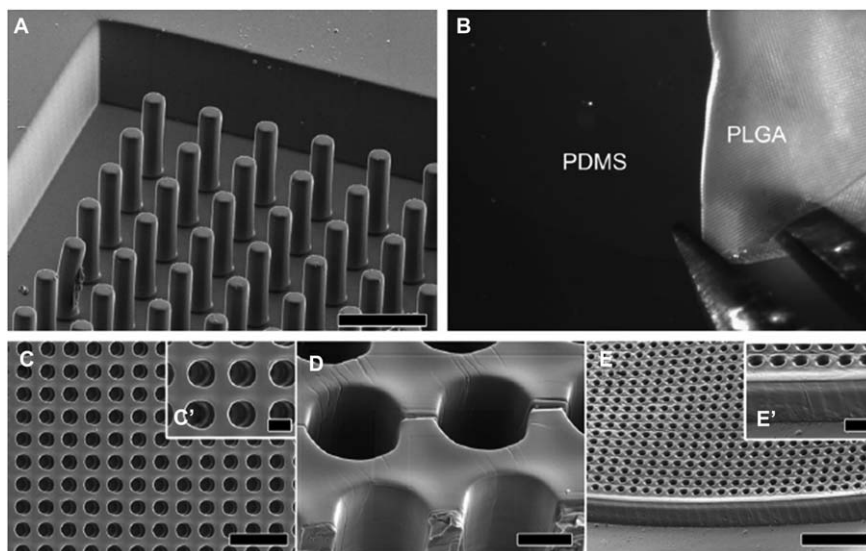


Figure 4.28 Fabrication of PLGA microchannel biomaterials using soft lithography. (A) Scanning electron micrograph (SEM) of micropillar negative mold in PDMS patterned from SU8/silicon master. (B) Patterning and removal of PLGA from PDMS-negative mold using forceps under ethanol. (C, D, E) SEM of top sides of resultant patterned PLGA microchannel scaffolds, exhibiting channel features that are 15 μm in diameter, 40 μm tall, and spaced 5 μm apart (edge-to-edge). (C) Overhead view. (C') Higher magnification of (C). (D) Alternate picture at 45° tilt angle with cutaway exhibiting inside walls of channels. (E) Alternate picture at 45° tilt angle exhibiting the edge of biomaterials. (E') Higher magnification of (E). Scale bars in (A) and (C) = 50 μm , (C') and (D) = 10 μm , (E) = 100 μm , and (E') = 20 μm .⁵⁵ Reproduced from ref. 55 with permission from Elsevier, Copyright 2012.

dissociated cells, which are induced from hPS cells, and provide the properties necessary for ideal *in vitro* models of retinal degenerative disease.

McUsic and his colleagues studied the reconstruction of organized retinal tissues using the Type B method described in Figure 4.3 by inoculating dissociated retinal progenitor cells obtained from hES cells into an array of aligned units that more precisely mimicked the retina.⁵⁵ This array of aligned units was generated by using soft lithography-patterned microchannels of PLGA to obtain geometric restriction (Figure 4.28). The PLGA scaffold had aligned microchannels, 15 μm in diameter, 40–50 μm thick, and spaced 5 μm apart (Figure 4.28A).⁵⁵ Cultivation of retinal cells in the scaffold at gas–liquid interface in low-serum medium enhanced viability of infiltrated rod photoreceptors 18-fold over high-serum cultivation when examined after 7 days.⁵⁵ Müller glia and rod photoreceptors developed from hES cells were aligned parallel to the patterned microchannel wall of the PLGA scaffold. When hES cell-derived retinal cells were cultivated in the microchannel PLGA scaffold, Pax6- (ganglion and amacrine cells existed in inner retina) and Otx2- (photoreceptor and bipolar cells of cones and rods in the outer nuclear layers) expressing cells were positioned in separate regions in normal retina (Figure 4.29).⁵⁵ These observation constitutes a significant advance in the creation of a tissue-level retinal model from dissociated retinal progenitor cells obtained from hPS cells that would be used in regenerative medicine or in drug screening platform.

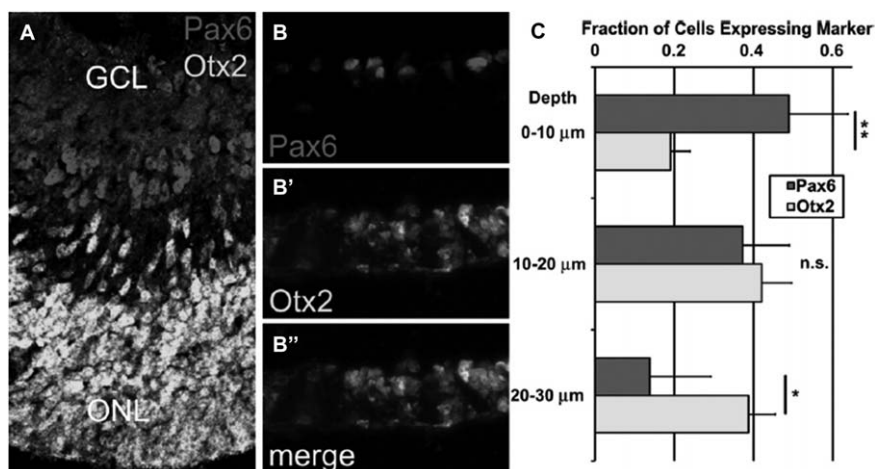
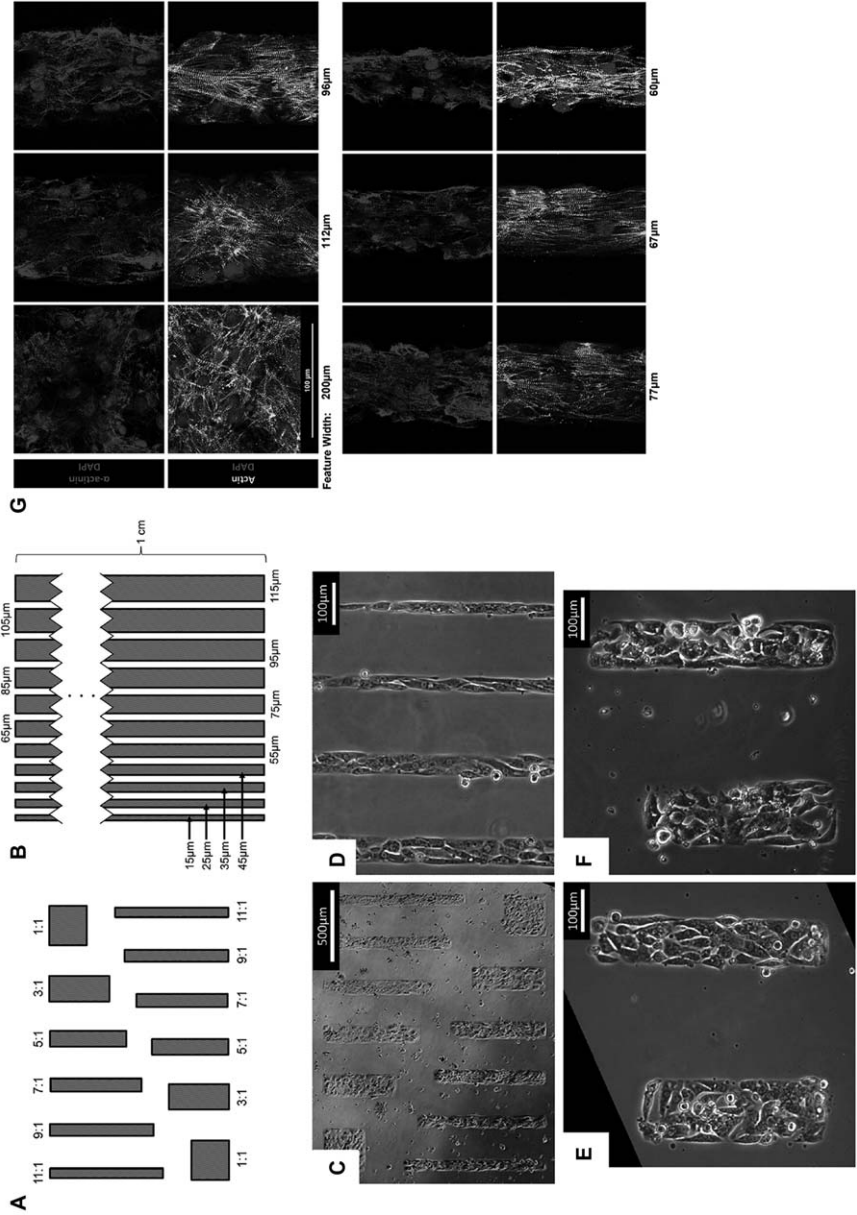


Figure 4.29 Lamination of dissociated mouse retinal cells cultivated in microchannel PLGA matrices. (A) Cryosection of a P6 mouse retina with the outer (apical) retinal surface down, exhibiting lamination of Otx2⁺ (green, outer retina) and Pax6⁺ (red, inner retina) cell populations. (B, B', B'') Cross-sections of dissociated neonatal mouse retinal cells cultivated in microchannel matrices for 3 days. (C) Binned-depth distribution of Otx2⁺ and Pax6⁺ cells in the matrices described as a fraction of all cells expressing the marker of interest.⁵⁵ Reproduced from ref. 55 with permission from Elsevier, Copyright 2012.



Salick and his colleagues studied sarcomere development in hES cell-derived cardiomyocyte cultivated on rectangular micropatterned ECM with some aspect ratios and micropatterned width using the Type H, G, and E methods described in Figure 4.3 (Figure 4.30).¹⁰⁴ This research was investigated because the aspect ratios and widths of rectangular micropatterns may guide the difference in costamere development, which in turn may influence sarcomere structure, polarization, alignment, and cell shape. They investigated whether rectangular micropatterned ECM regulated calcium propagation rates and altered gap junction plaque formation within cardiomyocyte aggregates on micropatterned surfaces and how multicell micropattern shapes played an important function in regulating sarcomere morphologies in hES cell-derived cardiomyocyte.¹⁰⁴ Figure 4.30A describes the rectangular micropattern designs with areas ranging from $160\,000\ \mu\text{m}^2$ to $2500\ \mu\text{m}^2$ and aspect ratios from 1:11 to 1:1, which were used in this research.¹⁰⁴

A microcontact printing protocol was used to make patterns of non-adherent PEG domains onto gold-coated glass plates. FN and Matrigel used as ECM components and were deposited onto the gold-coated glass plates, which form a regulated rectangular style for cell adhesion (Figure 4.30C–F). Then, hES cell-derived cardiomyocyte was inoculated onto the micropatterned ECM biomaterials, and the process of how cardiomyocyte deformed their myofilament structure in response to the micropatterned geometries of ECM was examined. Both the expression of phalloidin and α -actinin staining were examined by immunochemistry and were used to evaluate actin fibers in micropatterned cells (Figure 4.30G). Nuclear alignment was examined to study how cell directionality was affected by a micropatterned ECM biomaterials. hES cell-derived cardiomyocyte displayed clear alignment with the micropatterned morphologies that are dependent on its width rather than its aspect ratio.¹⁰⁴ Human ES cell-derived cardiomyocyte was found to extensively ordered on the micropatterned ECM biomaterials, having widths from $80\ \mu\text{m}$ to $30\ \mu\text{m}$, with an extensive enhancement in sarcomere alignment relative to the long axis of the micropatterned ECM biomaterials.¹⁰⁴ This design of extensively aligned cell aggregates with robust sarcomere structure should support to understand how

Figure 4.30 Micropattern width-dependent sarcomere development in hES cell-derived cardiomyocyte. (A, B) Micropattern illustration. ECM proteins were micropatterned into rectangles of varying aspect ratios and sizes. Two types of patterns were generated. (C–F) Representative brightfield pictures of micropatterned hES cell-derived cardiomyocytes. Cells inoculated onto micropatterned Matrigel–fibronectin designs matched the printed geometries. (G) Actin, α -actinin, and DAPI stains of cardiomyocytes after 5 days in cultivation on designs of varying widths. Both myofilament markers (actin and α -actinin) exhibit clearly improved sarcomere alignment, sarcomere organization, and cellular alignment in designs under $100\ \mu\text{m}$ in width. The cells inoculated onto square designs ($200\ \mu\text{m}$ in this set) do not favor any specific direction.¹⁰⁴ Adapted from ref. 104 with permission from Elsevier, Copyright 2014.

micropatterned ECM geometries give influence on myofilament maturation and structure when hES cell-derived cardiomyocytes are cultivated.

Axonal regeneration in CNS (central nervous system) is limited by the inhibitory influence of the glial and extracellular environment after CNS injuries.^{221,235} Implantation of NPCs (neural progenitor cells) and NS cells is an interesting idea for regenerating the damaged CNS. One instructive environment for restoration of function and axonal regeneration is the cultivation of NPCs, NS cells, or MS cell-derived neural cells on scaffold with guidance channel.^{202,221,222,236–239}

Ruiz and his colleagues developed PLL-micropatterned biomaterials coated with a plasma-polymerized PEO using the microcontact printing protocol.²⁰² Several patterns of 1 nm thickness cell adhesion PLL were prepared on a cell-repellent PEO materials. NS cells cultivated on the PLL patterns in induction media over 20 days showed excellent confinement to the PLL areas.²⁰² NS cells cultivated on the PLL-micropatterned biomaterials created random axon-like projection outside of the pattern and gave high amounts of neural marker expression in the induction media. Axon-like outgrowth and migration were extensively regulated by means of interconnected square pattern of PLL.²⁰²

Recknor and his colleagues examined directional differentiation and growth of AHP (adult rat hippocampal progenitor) cells on micropatterned PDMS surface coated with LN and PLL.²²¹ The micropatterned PDMS surface had striped groove shapes with 4 μm depths, 16 μm groove widths, and 13 μm terrace widths (Figure 4.23[f]). The micropatterned surface guided AHP cells into over 75% alignment in the groove direction.²²¹ AHP cells were also co-cultivated with astrocytes, producing almost twice the percentage of cells (35%) expressing TuJ-1 (class III β -tubulin) on the micropatterned biomaterials compared to AHP cells growing in the absence of astrocyte or on non-patterned surface.²²¹ These results indicate that a physical biomaterial cue (micropatterned biomaterials) in synergy with chemical (LN) and biological (astrocytes) leading cues promotes the neuronal differentiation of AHP cells.²²¹ Integration of these cues should be significantly important in controlling and understanding differentiation of NS cells and in designing scaffold for nerve generation in the future.

Microchannel surface patterned using photolithographic methods was also investigated to produce extensively oriented neurites as demonstrated previously.^{240–242} On the other hand, most of the research did not examine the effect of the micro-structure on stem cell induction into neural cells. Therefore, Beduer and his colleagues studied the effect of terrace and groove width of micropatterned surface with striped groove shapes on the induction of human NS cells into neurons. In this work, hNS cells were cultivated on the surface of micropatterned PDMS having different parameters of groove (g) and terrace (t) width: (a) $g = 60 \mu\text{m}$ and $t = 10 \mu\text{m}$, (b) $g = 20 \mu\text{m}$ and $t = 20 \mu\text{m}$, (c) $g = 10 \mu\text{m}$ and $t = 10 \mu\text{m}$, and (d) $g = 5 \mu\text{m}$ and $t = 5 \mu\text{m}$ (Figure 4.23[f]).²⁰¹ The micropatterned PDMS surface was coated with LN and PLL. A large majority of the adhesive cells developed neurites inside the microgrooves

along the walls and attached in the grooves.^{243,244} Neuronal induction as examined by Tuj-1 protein expression enhanced with the increase of groove width, whereas adhesive cell density was not dependent on either terrace or groove width.²⁰¹ The induction of stem cells into neurons was extensively influenced and reduced on the surface with micropatterned width less than the cell body diameter (e.g., 12 μm). Then, the size constraints imposed by the line microchannels of 20 μm –20 μm , 10 μm –10 μm , and 5 μm –5 μm led to an extensive reduction in the average number of neurites per neuron in comparison to the flat PDMS surface (control experiments).²⁰¹ The micropatterning of NS cells is found to affect the number of neurites per neuron. Neuronal cells extensively showed a single neurite (80–85%) when the cells were cultivated on micropatterned biomaterials with narrow grooves and terraces ($g = 5 \mu\text{m}$ and $t = 5 \mu\text{m}$), whereas only 25–29% of neural cells displayed single neurites on micropatterned biomaterials with wider grooves and terraces ($g = 60 \mu\text{m}$ and $t = 10 \mu\text{m}$).²⁰¹ The proportion of neurons having two or three neurites enhanced with the microchannel widths. Neurite length was influenced by the microchannel widths. An extensive reduction in neurite length was found on micropatterned biomaterials with narrow grooves ($g = 5 \mu\text{m}$ and $t = 5 \mu\text{m}$) in comparison to those micropatterned biomaterials wider grooves (e.g., $g = 20 \mu\text{m}$ and $t = 20 \mu\text{m}$ or $g = 60 \mu\text{m}$ and $t = 10 \mu\text{m}$).²⁰¹ Small micropatterns seemed to restrict neurite development.

Neural cells specifically aligned their neurites along the axes of the line pattern for neurite orientation and direction. Then, alignment was greater in the smaller microchannels. The proportion of neurites generating angles inferior to 10 degrees with the microchannel direction was 93–97% on the microchannel surface with narrow groove ($g = 5 \mu\text{m}$ and $t = 5 \mu\text{m}$), while that same proportion reduced to around 45% on the microchannel surface with wide groove ($g = 60 \mu\text{m}$ and $t = 10 \mu\text{m}$).²⁰¹ Micropatterned surface with narrow channel ($g = 5 \mu\text{m}$ and $t = 5 \mu\text{m}$) formed sharp neurite alignment, which is parallel to the microchannel direction, whereas the neurite length and differentiation were drastically restricted.

Nanotopography should affect stem cell induction into desired lineages, such as neural lineages (oligodendrocytes, astrocytes, and neurons), because ECMs *in vivo* have nanoscale topographies in stem cell niche. Yim and his colleagues cultivated hMS cells on nanopatterned and micropatterned PDMS surface with striped groove morphologies with (a) 350 nm depths, 10 μm groove widths, and 20 μm terrace widths, (b) 350 nm depths, 2 μm groove widths, and 20 μm terrace widths, (c) 350 nm depths, 1 μm groove widths, and 20 μm terrace widths, and (d) 350 nm depths, 350 nm groove widths, and 700 nm terrace widths.²²³ The nanopatterned and micropatterned PDMS surfaces were coated with collagen type I.

Human MS cell bodies and nuclei were extensively elongated, when hMS cells were cultivated on nanopattern surface with 350 nm groove widths in proliferation media where the groove size was one order of magnitude smaller than the cell body.²²³ F-actin fibers were extended extensively along the long axis of the cell. No alignment was found on unpatterned surfaces, whereas the

alignment of cells on nanopatterned surface was approximately 85%.²²³ Gene expression and microarray investigations indicated that muscular (myosin light chain and myf5) and neuronal (tyrosine hydroxylase [TH], neurofilament light peptide [NFL], and SOX2) gene expressions were extensively enhanced on nanopatterned biomaterials even in proliferation media, whereas the changes in gene expression were not almost found on micropatterned or unpatterned biomaterials.²²³ Especially, TuJ-1 and MAP2 (microtubule associated protein 2), which were mature neuronal markers, were also observed on nanopatterned biomaterials in proliferation media. A high terrace width dependency of neuronal induction was found on the patterned biomaterials.²²³ MAP2 expression enhanced with reducing groove width when hMS cells were cultivated in proliferation media. Synaptophysin expression was observed in hMS cells cultivated on nanopatterned biomaterials in differentiation and proliferation media but not on unpatterned biomaterials, indicating synapse generation in the cells cultivated on nanopatterned biomaterials.²²³

These investigations indicate that nanotopographies play an important function in controlling stem cell induction. Nanotopographies alone can regulate extensive enhancement of neuronal markers in hMS cells, indicating differentiation into the neuronal lineages.

4.3.3 Stem Cell Induction on Nanofibers

Stem cell cultivation on nanofiber is regarded as a sophisticated 3D culture of stem cells on nanopatterned biomaterials. Especially, structural protein fiber, such as native elastin and collagen in tissue, has diameter ranging from several dozen to several hundred nanometers.^{245,246} Nanoscaled protein fiber is entangled with each other and form non-woven protein fiber, generating elasticity and tensile strength in native tissue.²⁴⁵ Therefore, stem cell cultivation on nanofiber is regarded to mimic the stem cell environment *in vivo*. Four types of nanofibers are considered: (1) nanofiber generated by the self-assembly of amphiphile peptide molecules,^{236,245,247–254} (2) nanofiber generated by self-assembly of ECM such as collagen, (3) nanofiber formed by micro(nano)phase separation, (4) nanofiber generated by electrospinning.

Nanofiber made using the electrospinning protocol has large diameter on the upper end of that range, while nanofiber developed by the self-assembly of amphiphile peptide molecules has small diameters in the lower end of the range of natural ECMs such as collagens.²⁴⁷ Nanofiber formed using the microphase separation protocol has similar diameter to natural ECMs such as collagen and has macropore morphologies.²⁴⁷

4.3.3.1 Stem Cell Differentiation on Nanofiber Made by Self-assembly of Amphiphile Peptides

Self-assembling peptides generate nanofiber, which is able to be regulated at physiological pH by changing salt concentration.²⁴⁸ A transparent gel is generated by mixing cell cultivation media (DMEM supplemented with FBS)

containing peptide amphiphile. The transparent gel solution is consisted of nanofiber, which is examined scanning electron microscopy in dried samples^{236,245,247,248,255} and by AFM in solution.²⁵⁰ When these nanofiber hydrogels prepared by self-assembling peptides undergo shear thinning, nanofiber hydrogels immediately recover nearly 100% of their elastic modulus. Thus, there is a high probability that nanofiber hydrogels could be used as injectable delivery molecules for stem cells to the site of injuries *in vivo*.^{236,256}

Some kind of self-assembling peptides were designed, and several examples are summarized in Table 4.9.^{236,245,247–252} Self-assembling peptides have hydrophilic and hydrophobic domains to make amphiphilic properties

Table 4.9 Sequences of self-assembled amphiphile peptides for immobilization of stem cells.¹⁷ Adapted from ref. 17 with permission from American Chemical Society, Copyright 2013.

Name (model ECM)	Chemical sequence	Ref. (year)
PA-RGDS (collagen I, fibronectin, osteopontin)	CH ₃ (CH ₂) ₁₄ CONH-GTAGLIGQ- RGDS	251 (2009)
PA-DGEA (collagen I)	CH ₃ (CH ₂) ₁₄ CONH-GTAGLIGQ- DGEA	251 (2009)
PA-KRSR	CH ₃ (CH ₂) ₁₄ CONH-GTAGLIGQ- KRSR	251 (2009)
PA-RGES (dummy of RGDS)	CH ₃ (CH ₂) ₁₄ CONH-GTAGLIGQ-RGES	251 (2009)
PA-S (control)	CH ₃ (CH ₂) ₁₄ CONH-GTAGLIGQ-S	251 (2009)
IKVAV-PA (laminin)	IKVAV -Glu(E)-A ₄ G ₃ (CH ₂) ₁₅ CH ₃	236 (2004)
EQS-PA (control)	EQS -Glu(E)-A ₄ G ₃ (CH ₂) ₁₅ CH ₃	236 (2004)
RGD-PA (collagen I, fibronectin, osteopontin)	RGD -Glu(E)-A ₄ G ₃ (CH ₂) ₁₅ CH ₃	245 (2006)
RGD-PA (collagen I, fibronectin, osteopontin)	RGD -Glu(E)-A ₄ G ₃ (CH ₂) ₁₅ CH ₃	247 (2006)
RADA16-PDSGR (laminin)	Ac-(RADA) ₄ -GG PDSGR -CONH ₂	248 (2006)
RADA16-SDPGYIGSR (laminin)	Ac-(RADA) ₄ -GGSD PGYIGSR -CONH ₂	248 (2006)
RADA16-IKVAV (laminin)	Ac-(RADA) ₄ -GG IKVAV -CONH ₂	248 (2006)
RADA16-SKPPGTSS (bone marrow homing)	Ac-(RADA) ₄ -GG SKPPGTSS -CONH ₂	248 (2006)
RADA16-PFSSTKT (bone marrow homing)	Ac-(RADA) ₄ -GG PFSSTKT -CONH ₂	248 (2006)
RADA16-FLGFPT (bone marrow homing)	Ac-(RADA) ₄ -GG FLGFPT -CONH ₂	248 (2006)
RADA16-DGEA (collagen I)	Ac-(RADA) ₄ -GG DGEA -CONH ₂	248 (2006)
RADA16-RGDS (collagen I, fibronectin, osteopontin)	Ac-(RADA) ₄ -GG RGDS -CONH ₂	248 (2006)
RADA16-FPGERGVEGPGP (collagen I)	Ac-(RADA) ₄ -GG FPGERGVEGPGP -CONH ₂	248 (2006)
RADA16-PRGDSGYRGDSG (collagen VI)	Ac-(RADA) ₄ -GG PRGDSGYRGDSG -CONH ₂	248 (2006)
RADA16 (control)	Ac-(RADA) ₄ -CONH ₂	248 (2006)
MMP2-RGDS (collagen I, fibronectin, osteopontin)	Ac-GTAGLIGQ ERGDS	249 (2008)
MDP-RGDS (collagen I, fibronectin, osteopontin)	Ac-EESLSLSLSLS LEEGRGDS -CO-NH ₂	252 (2011)
RADA16-RGDSP (collagen I, fibronectin, osteopontin)	Ac-(RADA) ₄ - RGDSP	250 (2010)

for the formation of self-assembled nanofiber. The hydrophobic regions are typically consisted of RADA16 (*i.e.*, [RADA]₄), alkyl chain (*e.g.*, [CH₂]₁₅CH₃), or hydrophobic oligopeptides (*e.g.*, (Ala)₄(Gly)₃ [A₄G₃]) in most of self-assembling peptides,²³⁶ while the hydrophilic domains are made of cell receptor-binding peptides, such as YIGSR, IKVAV, KRSR, DGEA, RGDS, *etc.* Several self-assembling oligopeptides are also developed to own biodegradable properties. For this purpose, enzyme-degradable sites for GTAGLIGQ (Gly-Thr-Ala-Gly-Leu-Ile-Gly-Gln) and matrix metalloproteinase-2 (MMP-2), might be also added.²⁵¹ Table 4.10 shows some investigations on the induction of stem cells cultivated on nanofiber generated by the self-assembly of amphiphile peptide molecules.^{236,245,247,249–251,253}

Ikonen prepared six kinds of amphiphile peptide molecules, which could generate self-assembling nanofibers.¹²⁰ Figure 4.31 depicts six types of molecular structure of amphiphile peptide molecules where the nanofibers are generated using the self-assembly of amphiphile peptide molecules.¹²⁰

The differentiation of hES (H7) cells into cardiomyocytes was examined by co-culturing hES cells with END-2 cells using the Type F method described in Figure 4.3. Then, hES cell-induced cardiomyocytes were inoculated and cultivated in 3D and 2D self-assembling nanofiber hydrogels after dissociation of the cells. A pH-sensitive hydrophilic nanofiber hydrogel (type 1 in Figure 4.31) co-assembled with hyaluronic acid had good performance for the cultivation of hES cell-induced cardiomyocytes in 3D and 2D self-assembling nanofiber hydrogels.¹²⁰ Nanofibers made from the type 4 molecules described in Figure 4.31 alone could allow hES cell cultivation in 2D self-assembling nanofiber hydrogel, although hES cells in 3D self-assembling nanofiber hydrogel, which were made from type 4 did not extensively support hES cell cultivation. This is due to the fact that type 4 self-assembling nanofiber made degradation too fast to hold hES cells for long-term use.¹²⁰ hES cells cultivated on type 4 self-assembling nanofiber, which was co-assembled with hyaluronic acids retained inside of the nanofiber hydrogel, while hES cells cultivated on type 4 self-assembling nanofiber alone are easy to escape from the nanofiber hydrogel. Type 4 self-assembling nanofiber, which was co-assembled with hyaluronic acids could hold the beating ability of hES cell-induced cardiomyocyte for a long cultivation time in comparison to other kind of self-assembling nanofiber hydrogel.¹²⁰

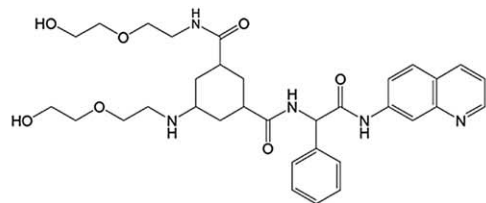
Anderson and his colleagues developed peptide amphiphile nanofiber inscribed with specific cellular adhesive ligands (*i.e.*, KRSR, DGEA, and RGDS) and examined whether the peptide amphiphile nanofiber could control osteogenic induction of hMS cells in the medium containing no osteogenic supplement.^{7,251} The peptide amphiphile nanofiber was used to develop self-assembled 2D coating on cell cultivation plates. Human MS cells cultivated on RGDS-grafting peptide amphiphile nanofiber, but not DGEA- or KRSR-grafting nanofiber, showed extensively higher ALP activity, suggesting early induction of osteoblast induction, and displayed a progressive shift into morphologies of osteoblast and high staining for mineral deposition.^{7,251} The peptide amphiphile nanofiber that mimics the native ECMs in bone was

Table 4.10 Some investigations into stem cell induction on nanofiber biomaterials made by self-assembled amphiphile peptides.¹⁷
Adapted from ref. 17 with permission from American Chemical Society, Copyright 2013.^a

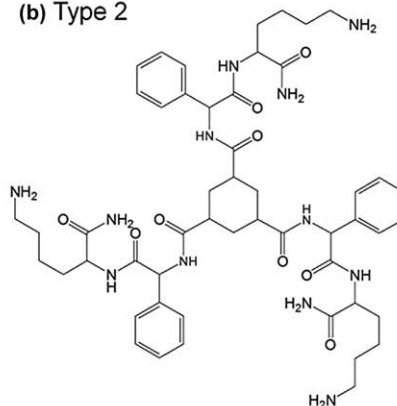
Stem cell source	Self-assembled peptide amphiphile for nanofiber preparation	Medium	Differentiation	Ref. (year)
hMSCs	Oligopeptides containing RGDS, DGEA, or GRES	Differentiation medium	Osteoblasts	251 (2009)
Rat MSCs	Oligopeptides containing RGDS	Differentiation medium	Osteoblasts	245 (2006)
Rat MSCs	Oligopeptides containing RGDS	Differentiation medium	Osteoblasts	247 (2006)
Human dental pulp stem cells, stem cells from human exfoliated deciduous supplements	Oligopeptides containing RGDS (GTAGLIGQERGDS)	Differentiation medium	Osteoblasts	249 (2008)
Rat marrow-derived cardiac stem cells	Oligopeptides containing RGDSP	Expansion medium	Cardiomyocytes	250 (2010)
Rat cardiac progenitor cells	Oligopeptides containing insulin-like growth factor-1	Expansion medium	Cardiomyocytes	253 (2009)
Murine neural progenitor cells	Oligopeptides containing IKVAV	Expansion medium	Neuronal cells	236 (2004)
Murine NSCs	Oligopeptides containing IKVAV, YIGSR, DGEA, RGDS, PDSGR	Differentiation medium	Neural cells	248 (2006)

^ahMSCs, human MSCs; MSCs, mesenchymal stem cells; NSCs, neural stem cells.

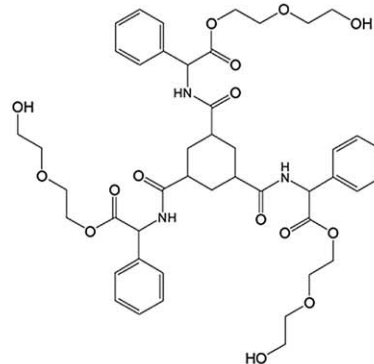
(a) Type 1



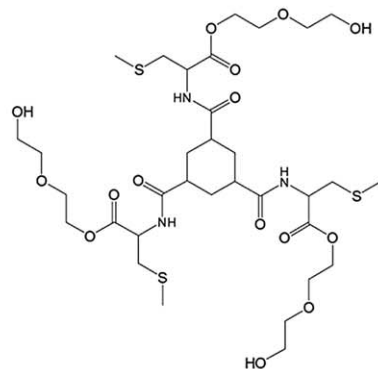
(b) Type 2



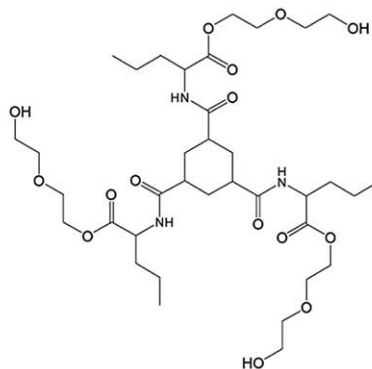
(c) Type 3



(d) Type 4



(e) Type 5



(f) Type 6

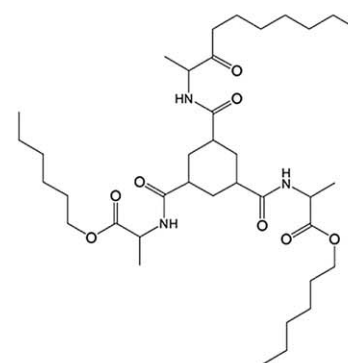


Figure 4.31 Molecular illustration of self-assembled amphiphile peptides. (a) Type 1, (b) Type 2, (c) Type 3, (d) Type 4, (e) Type 5, and (f) Type 6.¹⁸
Reproduced with permission from The Royal Society of Chemistry.

noticed to guide the osteogenic induction of hMS cells to a certain degree with no addition of supplements and gave an excellent environment, which allowed some adhesion molecules to regulate cellular behavior.^{7,251}

Silva and his colleagues studied a 3D network of nanofiber made by the self-assemblies of amphiphile peptide molecule (IKVAV-PA in Table 4.9) where NPCs were entrapped.²³⁶ The neurite-promoting LN epitope IKVAV (isoleucine-lysine-valine-alanine-valine) was contained in the amphiphile peptide molecule. Self-assemblies were triggered by mixing amphiphile peptide molecule and cell suspension in medium. The resulting self-assembled nanofiber immobilized the bioactive epitope (IKVAV) on their surface at van der Waals packing distances and formed a gel-like solid having 99 wt% water.²³⁶ The nanofibers had large surface areas and high aspect ratios, 5–8 nm in diameter and lengths ranging from a few micrometers to hundreds of nanometers. Therefore, the nanofiber could show the IKVAV epitope to neural progenitors at an extensively high density relative to natural ECM, LN.²³⁶

Cell body area and neurite length within the nanofiber network was noticeably larger than those in neurons cultivated on 2D surface.²³⁶ NPCs induced into neurons on the self-assembled nanofiber scaffold, in contrast to cells cultivated on LN-coated or PDL (poly-D-lysine) coated plates, which reduced astrocyte induction.²³⁶ We observed that the physical immobilization of IKVAV in the self-assembled nanofiber, not solely its presence in the scaffolds, was a key point to the neuronal induction of neural progenitors. This is because the immobilization of IKVAV-soluble peptides into hydrogels entrapped with neural progenitors where the IKVAV sequence, which was modified into the non-bioactive peptide of EQS (glutamic acid-glutamine-serine) did not facilitate selective neuron induction.²³⁶

Gelain and his colleagues examined a 3D network of nanofibers where NPCs were entrapped *in vitro*, which generated the self-assemblies of amphiphile peptide molecule with several bioactive peptides, including cell adhesion (FPGERGVEGPGP from collagen type I, RGDS from FN, and IKVAV and SDPGYIGSR from LN), differentiation, and bone marrow homing (FLGFPT, PFSSTKT, and SKPPGTSS) motifs.²⁴⁸ The peptide amphiphile nanofiber hydrogels with bone marrow homing peptides (PFSSTKT and SKPPGTSS) increased survival of neural cells without addition of neurotrophic factors and/or soluble growth factors in the cultivation media.²⁴⁸ The populations of Nestin⁺ (neural progenitor) (superscript of “+” means the expressing of this gene or protein), GFAP⁺ (astrocyte), and β -III tubulin⁺ (neuron) cells cultivated on peptide amphiphile nanofiber hydrogels with the optimal peptide sequences were extensively higher than the populations in common 2D cultivation (*i.e.*, TCP dishes) and were similar to the populations cultivated on Matrigel-coated dishes.²⁴⁸ Matrigel is consisted of isolated components from the sarcomas of Engelbreth-Holm-Swarm mice,^{257,258} containing growth factors (*e.g.*, TGF- β , EGF, and FGF), enactin, heparan sulfate proteoglycans, LN, and collagen type IV. Matrigel includes unknown ingredients and is isolated from mice. However, synthetic oligopeptide amphiphiles are chemically defined molecules, which are a key

point for the use of stem cells and/or scaffolds in clinics, although Matrigel is an useful biomaterial to guide the appropriate differentiation of stem cells or to support pluripotent state of stem cells.^{2,259,260} Self-assembling peptide nanofiber hydrogels made by Gelain and his colleagues could mimic the properties of Matrigel to regulate stem cell induction into appropriate lineages of the cells.²⁴⁸ They succeeded in regulating NS cells into glial and neural induction without the extra addition of growth factors by immobilizing the NS cells in self-assembled peptide amphiphile nanofiber hydrogels.

Hosseinkhani and his colleagues fabricated a 3D network of nanofibers generated by the self-assemblies of amphiphile peptide molecules including RGD peptide (RGD-PA in Table 4.9) where rat MS cells were entrapped.²⁴⁷ 3D nanofiber networks were also generated in hydrogels by blending MS cell suspensions in medium with dilute aqueous solution of the oligopeptide amphiphile molecules. The expansion, attachment, and osteogenic induction of MS cells were successfully promoted in the oligopeptide amphiphile nanofiber hydrogels without and with RGD peptide in comparison to MS cells in traditional 2D cultivation on cell cultivation plates.²⁴⁷ The addition of the RGD peptide in the self-assembled amphiphile nanofiber led MS cells to facilitate higher expansion, attachment, and osteogenic induction in comparison to those without the RGD sequence. This result is elucidated by the ability that binds to MS cell integrin receptors with the RGD on the oligopeptide amphiphile promoted cell adhesion, along with expansion and osteogenic induction, on the nanofiber.²⁴⁷

The 3D matrices used in traditional tissue engineering require surgery for their transplantation, which is not really desirable for clinical treatment. Gel scaffold composed of nanofiber networks generated by the aggregation of the oligopeptide amphiphile, and the procedure can be physically triggered by the addition of a cell suspension to the aqueous oligopeptide amphiphile solution. The hydrogels generated by this procedure could be delivered to injured tissue by injection of the combined peptide amphiphile solution and cell suspension, which allow the injected solution to generate a hydrogel at the injection domain.^{247,249}

Self-assembling oligopeptide nanofiber hydrogels should be valuable for the 3D cultivation of stem cells in regenerative medicine and in general molecular and cellular biology.

4.3.3.2 *Stem Cell Differentiation on Nanofibers Generated by Electrospinning Methods*

Electrospinning nanofiber can be fabricated from a spinning nozzle when high voltage is applied between a flat metal collector and spinning nozzle. Some types of nanofiber morphologies can be formed with the electrospinning process, such as structures resembling cotton balls, oriented fabric-like sheets, and non-woven fabric-like sheets. The general electrospinning methods are schematically depicted in Figure 4.32.

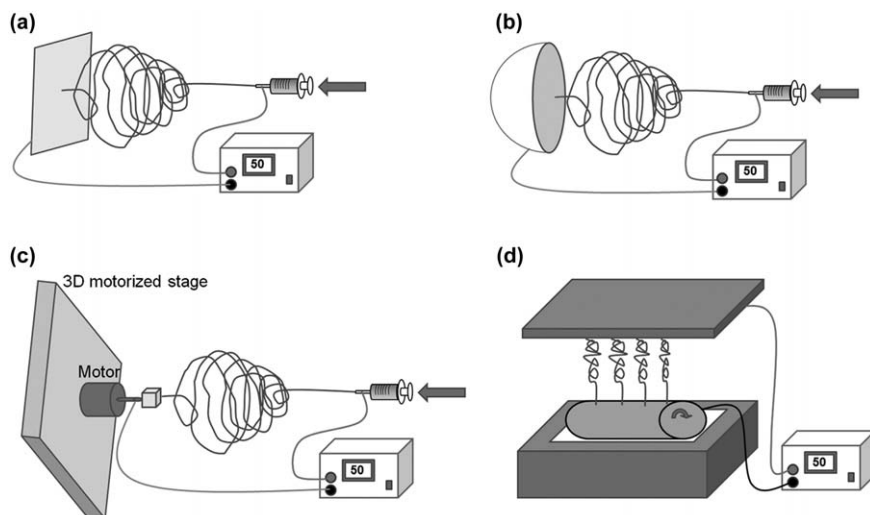


Figure 4.32 Some illustrations of electrospun methods. Traditional electrospun method to generate non-woven fabric scaffold (a), the electrospun method to generate cotton ball-like scaffold (b), the electrospun method to generate double-layered 2D architecture (crosshatch pattern) using a 3D stage (c), and the Nanospider™ electrospun method (d).¹⁷ Reproduced from ref. 17 with permission from the American Chemical Society, Copyright 2013.

Aligned nanofibers generated using the electrospinning protocol have been reported (Figure 4.32C). The rotating fiber collector makes nanofiber to order with one another. Double-layer 2D architecture (crosshatch pattern) are achieved by orthogonal substrate orientation and repeated nanofiber deposition.

Electrospun fibers are flat and extensively interconnected scaffold with a sheet-like and non-woven fabric morphologies in typical cases.⁷ These properties restrict cell growth and infiltration throughout the scaffolds. Blakeney and his colleagues prepared a 3D cotton ball-like electrospun scaffolds, which were uncompressed nanofibers with low-density.²⁶¹ An array of needle-like probes and a grounded spherical plate were used instead of a typical flat plate collector to form cotton ball-like scaffolds (Figure 4.32B).⁷ Scanning electron microscopy showed that the cotton ball-like scaffolds composed of electrospinning nanofiber with similar diameter but bigger pore size and less-dense morphologies than typical electrospinning scaffolds.²⁶¹ These cotton ball-like morphologies should be valuable for use as matrices for regulating some differentiation lineages of stem cells.

One of the disadvantages of nanofiber production using the electrospun protocol is the extensively low spinning speed. To solve this issue, a rotating metallic drum dipping into polymeric solution was considered as a spinning nozzle to generate multiple nanofibers from the drum not using a single-nozzle spinning needle (Nanospider™, Figure 4.32D).²⁶² In the future, this

new method might contribute to the spinning of nanofiber scaffolds on an industrial level.

Table 4.11 shows some nanofibers produced using the electrospun protocol for stem cell induction, which have been investigated by several researchers.^{51,163,255,263–289}

Typically, hMS cells are difficult to induce into chondrocyte in 2D monolayer cultivation. Hanging drop and/or pellet cultivation of hMS cells are the typical protocols for chondrogenic induction.²⁹⁰ This is explained as high cell densities guide higher chondrogenic induction. Cell–cell contact and autocrine growth factors are critical in chondrogenic differentiation. Condensation of hMS cells triggers chondrogenic differentiation during skeletal development,²⁹¹ which indicates the rationale in high-density pellet cultivation for chondrogenic differentiation.^{217,218,292,293} Moreover, the cell morphologies in hanging drop and pellet cultivation is round, which is opposite to spreading, as is found in monolayer cultivation. Morphological guidance seems to be also a key factor facilitating chondrogenic differentiation of hMS cells.⁷

Nanofibers prepared using the electrospinning method show high surface area to volume ratio, which generate high cell–material contact. Several researchers showed that hMS cells on electrospinning nanofiber is able to induce into adipocytes, osteoblasts, and chondrocytes.^{263,267,270,274,278} Xin investigated that hMS cells could induce into both osteoblasts and chondrocytes, which depend on the induction medium, when the cells were cultivated on PLGA nanofiber. These results are valuable for stem cell application of osteoarthritis due to the continuous induction of hMS cells into chondrocytes and osteoblasts.²⁷⁰

At this moment, an optimal method to induce hepatic differentiation in hPS cells has been developed. However, the effects of biomaterial and ECM on hPS cells remain unclear, which are being cultivated for induction into hepatocytes. Therefore, Yamazoe and his colleagues developed a protocol of hepatic differentiation from hPS cells and mES cells, which were cultivated on commercially available electrospinning nanofiber (UltraWeb, 280 nm diameter) composed of polyamide using the Type E method described in Figure 4.3. They compared the growth on the nanofibers with the growth on several 2D biomaterials (TCP plates coated with CellStart, Matrigel, gelatin, or collagen type I).¹⁰⁶ The Type E differentiation method (Figure 4.3), which is similar to preparation methods examined by Ramasamy and his colleagues¹⁰¹ and Farzaneh and his colleagues,⁸⁴ was used for the hPS cell differentiation. When ES cells were differentiated into endoderm, followed by differentiation into hepatocytes on the nanofiber or on some other biomaterials, characteristics evaluation of hepatocytes indicated that ES cells cultured on nanofibers coated with Matrigel were more extensively facilitated toward induction into hepatocytes than hES cells cultivated on other 2D biomaterials.¹⁰⁶ Rac1 activation was detected in ES cells cultivated on the nanofiber in comparison to the ES cells cultivated on other biomaterials where Rac1 is a Rho family member and regulates the actin cytoskeleton.

Table 4.11 Some investigations into stem cell induction on nanofibers made by the electrospun method. Adapted from ref. 17 with permission from American Chemical Society, Copyright 2013.^a

Stem cell source	Materials for stem cell culture	Medium	Differentiation	Ref. (year)
hMSCs	PCL nanofibers	Differentiation medium	Osteoblasts, chondrocytes, adipocytes	274 (2005)
hMSCs	PLLA nanofibers	Differentiation medium	Osteoblasts	276 (2005)
hMSCs	Non-woven collagen type I nanofibers (diameter (d) = 50–200, 200–500, and 500–1000 nm)	Differentiation medium	Osteoblasts	263 (2006)
hMSCs	Nanofibers composed of nano-sized demineralized bone powders with PLLA composite material	Differentiation medium	Osteoblasts	264 (2008)
hMSCs	BMP-2-incorporated PLLA nanofibers	Differentiation medium	Osteoblasts	244 (2008)
hADSCs	Collagen type I nanofibers	Differentiation medium	Osteoblasts	265 (2008)
hMSCs	PLLA-collagen I blend nanofibers	Differentiation medium	Osteoblasts	266 (2009)
hUSSCs	Plasma-treated PLLA nanofibers coated with nanohydroxyapatite	Differentiation medium	Osteoblasts	267 (2010)
hMSCs, hAFSCs	PCL nanofibers	Differentiation medium	Osteoblasts	275 (2010)
Rabbit MSCs	Nanofibers composed of nano-sized hydroxyapatite and PCL having 340 nm diameter	Differentiation medium	Osteoblasts	268 (2011)
hUSSCs	Plasma-treated or collagen-grafted PES nanofibers	Differentiation medium	Osteoblasts	269 (2011)
hMSCs	Non-woven PLGA nanofibers with a diameter of 760 nm	Differentiation medium	Osteoblasts, chondrocytes	270 (2007)
MSCs	PLA nanofibers	Differentiation medium	Chondrocytes	278 (2008)
Calf MSCs	PCL nanofibers	Differentiation medium	Fibrocartilaginous cells	271 (2011)
hMSCs	Aligned and randomly oriented nanofibers prepared from thermally responsive hydroxybutyl chitosan	Expansion medium	Myocytes	272 (2007)
hATSPCs	Aligned and randomly oriented PLLA nanofibers	Expansion and differentiation medium	Tendon	273 (2010)
Murine ESCs (CE3, RW4)	Aligned and randomly oriented PCL nanofibers	Differentiation medium	Neural cells	287 (2009)

Table 4.11 (Continued)

Stem cell source	Materials for stem cell culture	Medium	Differentiation	Ref. (year)
hMSCs	PLCL/collagen nanofibers	Differentiation medium	Neural cells	279 (2009)
Rat ANSCs	PLO and laminin-coated PCL nanofibers by electrospinning ($d = 260, 480, 930$ nm)	Differentiation medium	Neural cells	255 (2010)
Mouse NSCs (C17.2)	Collagen nanofiber crosslinked with rose bengal as photoinitiator by laser irradiation	Expansion medium	Neuronal cells	280 (2010)
Rat NSCs	Aligned, single- and double-layer polystyrene nanofiber meshes coated with PLO and laminin ($d = 800$ nm)	Expansion medium	Neuronal lineages	289 (2011)
hESCs	PLO/laminin-coated PCL	Expansion medium	Neural cells	281 (2011)
hMSCs	PCL-gelatin nanofibers immobilized retinoric acid ($d = 240$ – 280 nm)	Expansion medium	Neural cells	288 (2012)
hESCs	Tusan silk fibroin nanofibers coated with poly-D-lysine (PDL)/laminin ($d = 400$ and 800 nm)	Differentiation medium	Neuronal cells	51 (2012)
UCBPCs	Aminated PES nanofibers	Differentiation medium	Endothelial and smooth muscle cells	282 (2009)
Rat MSCs	Photopolymerized PEG nanofibers coated with collagen type I	Expansion medium	Endothelial and smooth muscle cells	163 (2012)
Rat MSCs	PLGA and collagen nanofibers immobilized CD29 antibody	Expansion medium	Epidermal cells	283 (2011)
hUSSCs	Oxygen-plasma treated nanofibers of poly(ϵ -caprolactone)	Differentiation medium	Hepatocytes	284 (2009)
hMSCs	Collagen-grafted PLLA nanofibers	Differentiation medium	Hepatocytes	285 (2012)
Murine limbal stem cells, MSCs	Polyamide-6/12 nanofibers by electrospinning ($d = 290$ – 539 nm)	Expansion medium	Proliferation and transplantation into damaged ocular surface	286 (2012)

^aANSCs, adult neural stem cells; ESCs, embryonic stem cells; hADSCs, human adipose-derived stem cells; hAFSCs, human amniotic fluid stem cells; hATSPCs, human fetal Achilles tendon stem/progenitor cells; hESCs, human ESCs; hMSCs, human MSCs; hUSSCs, human unrestricted somatic stem cells (stem cells from UCB); MSCs, mesenchymal stem cells; NSCs, neural stem cells; PES, polyethersulfone; PLC, poly(ϵ -caprolactone); PLCL, poly(L-lactic acid)-*co*-poly-(3-caprolactone); PLGA, poly(lactic acid-*co*-glyconic acid); PLLA, poly(L-lactic acid); PLO, poly-L-ornithine; UCBPCs, UCB-derived progenitor cells (CD133⁺ cells).

Inhibition of Rac1 restricted endoderm and hepatic induction of ES cells cultivated on the nanofiber throughout any induction stages. The morphological changes of ES cells cultivated on nanofiber indicated the cellular differentiation regulated by Rac1 activation.¹⁰⁶ The nanofiber could recapitulate the structural features of the stem cell niche that regulated the induction of hPS cells into desired cell lineages and contribute to drug delivery and regenerative medicine.

4.3.3.2.1 Size Effect of Nanofibers on Stem Cell Induction. Shih and his colleagues made collagen type I nanofiber having different diameters (500–1000, 200–500, and 50–200 nm) using the electrospinning nanofibers upon which hMS cells were inoculated and investigated for cell motility, adhesion, growth, and osteogenic induction.²⁶³ Cells having all nanofiber sizes had more flattened and polygonal cell shapes than the cells on TCP plates. Furthermore, hMS cells proliferated on 500–1000 nm nanofibers had much better viability of the cells than TCP plates.²⁶³

Christopherson and his colleagues examined the impact of nanofiber size on the differentiation of adult rat hippocampal-derived NS cells.^{289,294} They recognized that NS cells cultivated on smaller diameter (280 nm) fibers induced selectively into oligodendrocyte precursors in medium containing retinoic acid and serum, whereas NS cells extensively induced into neuronal precursors on fibers having larger diameter (750 nm).

4.3.3.2.2 Effect of Nanofiber Alignment on Stem Cell Induction. Bakhru and his colleagues made double-layer (crosshatch pattern), single-layer (uniaxially aligned) and highly aligned polystyrene nanofiber meshes for the investigation of NS cell fate, which is affected by the physical microenvironment of the cells. Aligned nanofibers coated with LN and PLO (poly-L-ornithine) induced cellular elongation and polarized NS cell morphologies in the direction of fiber alignment, which are key points for NS cell neuronal induction.²⁸⁹ NS cells on conventional flat TCP plates specifically induced into astrocytes (glia) and not into neuronal lineages (efficiency of less than 10%), whereas the aligned fiber meshes facilitated NS cell neuronal lineage induction with an efficiency of 83%.²⁸⁹ This study indicated that microenvironmental physical cues of biomaterials decide stem cell fate of induction.

Lam and his colleagues examined the effect of random and aligned nanofibers on induction of hES cells into neural cells using the Type D and B protocols (Figure 4.3).⁴⁹ PLLA nanofiber scaffold was generated by an electrospinning protocol. Axon growth is a key point during neural tissue regeneration. Then, the effect of random and aligned nanofibers on axon growth was examined using ES cell-derived neural cells that were made from selection of rosettes using EB formation and were cultivated on random and aligned PLLA nanofiber scaffold coated with LN and PLO for 7 days. LN and PLO are typically used as surface coatings to enhance the adhesion of neural cells.⁴⁹

Axons were randomly generated and short on random nanofiber scaffold and on typical TCP plates coated with LN and PLO, while axons aligned and

grew in the direction of aligned nanofiber on aligned nanofiber scaffold.⁴⁹ An aligned nanofibrous morphology seemed to be preferable for extended axon outgrowth in this research.

Heparin-conjugated PLLA nanofiber scaffold was generated and FGF-2 or EGF was subsequently sorbed onto the nanofiber scaffold to combine physical cues (aligned nanofibers) and biochemical cues (EGF and FGF-2; growth factors).⁴⁹ This is because heparin can bind to these growth factors. For preparation of heparin-grafted nanofibers, PLLA nanofiber scaffold was inserted in NaOH solution for saponification to create carboxylic acid, and subsequently, heparin was covalently conjugated to the nanofiber by the crosslinking agents *N*-hydroxysulfosuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride for generation of amide bonding. The scaffolds were coated with LN and PLO, after immersion of the heparin-grafted nanofiber scaffold into either an EGF or FGF-2 solution to make the growth factor binding to heparin.⁴⁹

Rosettes were derived from hES cells through EB generation and were subsequently inoculated and cultivated on the FGF-2- or EGF-sorbed PLLA nanofiber scaffold and heparin-immobilized PLLA nanofiber scaffold sorbed with FGF-2 or EGF for 21 days, at which point all of the nanofiber scaffold was coated with LN and PLO. Axon outgrowth from rosettes did not indicate any significant difference after EGF or FGF-2 were sorbed onto the PLLA nanofiber scaffold. However, the immobilization of EGF or FGF-2 onto PLLA nanofiber scaffold, which was immobilized with heparin extensively facilitated axon growth, suggesting that the bioactivity of EGF and FGF-2 was retained *via* heparin binding but not by sorption onto the PLLA nanofiber scaffold.⁴⁹

EGF and FGF-2 were ineffective in induction of axon growth if these growth factors were directly sorbed onto PLLA nanofiber scaffold.⁴⁹ This is explained as passive sorption on PLLA nanofiber scaffold leads to deformation of protein conformation, which should decrease the activities of the sorbed growth factors on the nanofiber scaffold. The binding of heparin onto the nanofiber scaffold makes the conjugation of EGF and FGF-2 to heparin in their native conformation, which sustains native activities and enhance half-lives of the growth factors. Because the nanofiber scaffold has extensively larger surface area than the area of typical TCP flat plates, the nanofiber morphology enhances the loading amount of growth factors. Furthermore, an aligned nanofiber morphology promotes interaction with receptors on the surface of axon together with the immobilized growth factors when the axon grows along the aligned nanofibers.⁴⁹ The combination of neurotrophic factors conjugated on the nanofiber scaffold and aligned nanofiber morphology should have significant function for tissue engineering application of neural cells.

Mahairaki and his colleagues prepared electrospinning PCL nanofibers and microfibers having several orientations (*i.e.*, random and aligned) and some diameters (microfibers of 1 μm diameter and nanofibers of 250 nm diameter), which were coated with LN and PLO to enhance cell attachment,

and examined hES cell differentiation into neurons, which were cultivated on these fibers.²⁸¹ They found that neural precursors cultivated on random fiber matrices or on plain TCP surfaces generated nonpolarized neurite networks, while hES cell-derived neural precursors adopted polarized cell morphologies having extension along the axes of fibers aligned with fibers. hES cell-derived neural precursors cultivated on aligned fiber matrices showed a higher ratio of neuronal induction than the neural precursors cultivated on other matrices; approximately 85% and 60% of the hES cell-derived neuronal precursors showed β III tubulin⁺ (early neurons) expression when cultivated on aligned nanofibers and microfibers, respectively, whereas only 25% and 30% were β III tubulin⁺ expression when cultivated on random nanofiber matrices and microfiber matrices, respectively.²⁸¹ It is suggested that hES cell-derived neural precursors cultivated on aligned nanofibers undergo neuronal differentiation, which seems to be independent on coating materials or nanofiber materials, as reported in work by Lam and his colleagues,⁴⁹ Mahairaki and his colleagues,²⁸¹ and Wang and his colleagues.⁵¹

Wang and his colleagues formed electrospinning nanofiber scaffold using Tussah silk fibroin (TSF), which had several diameters (800 and 400 nm) and aligned or random orientations. They cultivated hES cells on the scaffold to investigate the effects of fiber alignment and fiber diameter on neurite outgrowth and neuronal differentiation in hES cell-derived neural precursors using the Type D and B protocols (Figure 4.3).⁵¹ TSF-nanofiber scaffold was found to support the differentiation, migration, and survival of hES cell-derived neural precursor. The aligned TSF-nanofiber scaffold especially promoted neurite outgrowth and neuronal differentiation in hES cell-derived neurons in comparison to the random TSF-nanofiber scaffold, which was similar to the findings reported by Lam and his colleagues.⁴⁹ Furthermore, neurite outgrowth and neuronal differentiation on aligned nanofibers of 400 nm diameter were higher than those on aligned nanofibers of 800 nm diameter.⁵¹ These results indicate that the aligned TSF-nanofiber scaffold of 400 nm diameter seems to be excellent for the development of hES cell-derived neural precursor.

Ren and his colleagues also studied the effect of fiber morphology on the induction of hES cell-derived NCSCs towards a Schwann cell lineage.²⁹⁵ They made electrospinning polyethersulfone micro/nanofibers having several alignments and diameters, which were coated with Matrigel. They found that aligned fiber scaffold extensively promoted cell alignment and that fibers with average diameters of 1600 nm and 600 nm greatly facilitated the induction of NCSCs towards a Schwann cell lineage in comparison to aligned fibers of 160 nm diameter and random fibers having diameters of 160–1600 nm as well as 2D TCP dishes.²⁹⁵ These results indicated that the fate of differentiation lineage in hPS cells relies not only on fiber morphology (*e.g.*, random or aligned micro/nanofibers) but also on the diameter of electrospinning fibers. The micro/nanofibers having diameters of more than 500 nm facilitate differentiation of stem cells, especially hPS cell-derived

neural precursors into Schwann cells, whereas fibers having diameter <500 nm facilitate the differentiation into neuronal lineages.

Xie and his colleagues differentiated mouse ES cells into NPCs by adding retinoic acid into the medium generating EBs. They investigated biodegradable PCL nanofiber matrices inoculated with NPCs and observed that EBs cultured on uniaxially aligned PCL nanofiber promoted induction into neural lineages and facilitated neurite outgrowth comparing with EBs cultivated on randomly oriented PCL nanofiber.²⁸⁷ Neurites cultivated on randomly oriented nanofiber were found to extend in all directions, whereas Neurites induced from EBs on aligned nanofiber showed extension along the direction of nanofiber alignment. Less astrocytes were observed on aligned nanofibers than on randomly oriented nanofibers.²⁸⁷ The maximum length of neurite projections from EBs cultivated on aligned nanofiber was extensively higher (>500 μm) than that of neurites on randomly oriented nanofiber.²⁸⁷ Aligned nanofiber was found to facilitate both the rate of neurite outgrowth direction and extension.

Lim and his colleagues also found that adult rat NS cells on aligned PCL nanofiber matrices coated with LN and PLO promoted higher neuronal differentiation in comparison to NS cells on randomly aligned PCL nanofiber or unpatterned matrices.²⁵⁵ Aligned nanofiber matrices of 480 nm diameter showed the highest fraction of neural progenitor in comparison to nanofibers with 260 or 930 nm diameter. This result is probably explained by neuron matrix selectivity, which indicates that aligned fiber matrices were less receptive to the attachment and survival of oligodendrocytes than unpatterned matrices or randomly oriented fibers.²⁵⁵

Leung and his colleagues⁸⁷ investigated a myogenic induction protocol for hES cells (BG01v), which were cultivated on aligned chitosan-polycaprolactone (CS-PCL) nanofiber using the Type E method (Figure 4.3). The nanofiber was designed to mimic the microenvironment of native muscle ECMs in concert with the Wnt3a protein but without cocultivation of feeder cells or other source of cells.⁸⁷ PCL was chosen as a biocompatible and synthetic polymer with excellent mechanical stability *in vivo*, whereas chitosan (CS) was chosen because CS is a natural and biodegradable polysaccharide, which is prepared by the partial deacetylation of chitin and have structural similarity to glycosaminoglycan existed in native ECMs.²⁹⁶ An electrospinning technique was used to make both aligned CS-PCL nanofibers (180 nm diameter) and random CS-PCL nanofibers (215 nm diameter) as well as PCL nanofibers. hES cells were cultivated on collagen type I film, CS-PCL film, random PCL nanofiber, aligned and random CS-PCL nanofiber in myogenic induction medium.⁸⁷ Myogenic induction was examined by myogenic marker protein expression, myogenic gene expression, and cell morphology. The ratios of cells showing the myogenic protein MyoD expression in induced hES cells cultivated on each material surface for 2 day culture were evaluated by flow cytometry (surface marker examination of the cells) and the results are described in Figure 4.33.⁸⁷ The induced hES cells, which were cultivated on collagen type

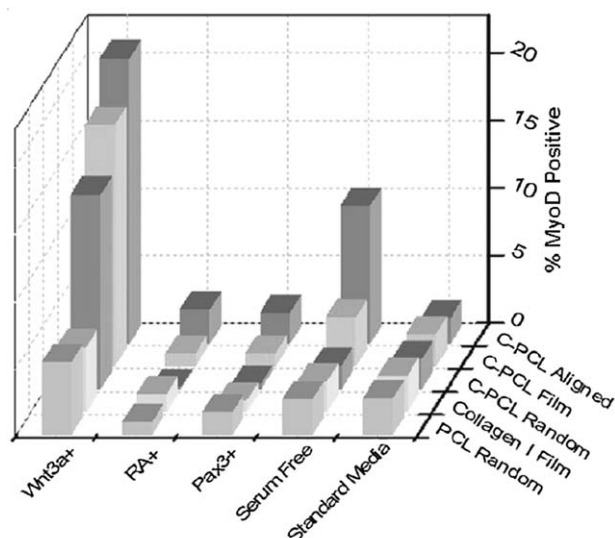


Figure 4.33 Myogenic induction of hES cells examined by flow cytometry. (a) Populations of hES cells expressing MyoD after 2 days in cultivation on random or aligned chitosan-polycaprolactone (C-PCL) nanofibers (C-PCL random and C-PCL aligned, respectively), C-PCL film, PCL random nanofibers (PCL random) and collagen type I-coated dishes (collagen I film) in medium including some growth factors [Pax3, Wnt3a, and retinoic acid (RA)].⁸⁷ Reproduced from ref. 87 with permission from American Chemical Society, Copyright 2013.

I film and random PCL nanofibers expressed the lowest levels of MyoD (myogenic marker). Aligned CS-PCL nanofiber was shown to be an excellent cultivation biomaterial for the induction of hES cells into myocyte (Figure 4.33).⁸⁷

Moreover, induced hES cells expressed higher MyoD when hES cells were cultivated in medium including Wnt3a protein than in any type of medium without Wnt3a protein on any biomaterials studied in this research (Figure 4.33). Wnt3a is known to play important functions in controlling myogenesis²⁹⁷ and in inducing epaxial myotome myogenesis that guides to skeletal muscle generation during embryonic myogenesis.²⁹⁸ hES cells cultivated on CS-PCL uniaxially aligned nanofiber in medium including Wnt3a protein generated elongated morphologies and were homogeneously aligned in the direction of fiber orientation, with enhanced expression of myogenic marker proteins (My5, Myf6, myogenin, and MHC) and myogenic marker genes (MHC, myogenin, MyoD, My6, My5, Pax7, and Oax3) in comparison to hES cells cultivated on control biomaterials.⁸⁷ The combination of aligned CS-PCL nanofibers (physical cues) and Wnt3a signaling (biological cues) lead to high percentages of myogenic protein-expressing cells relative to total treated hES cells (85% expressed My5, 90% expressed Myf6, 65% expressed

MHC, and 85% expressed myogenin) after 48 h in cultivation.⁸⁷ The CS-based aligned nanofiber in this research combined with Wnt3a protein may potentially serve as a good model system for muscle regeneration and embryonic myogenesis.

There are several studies, which examined the effects of the diameter of aligned and random microfibers and nanofibers on the induction of hPS cells into several cell lineages followed their cultivation on these fibers.^{83,281} hES cells cultivated on aligned PCL fibers having 500 nm diameter were found to promote chondrogenic induction in induction medium in comparison to hES cells cultivated on aligned microfiber having 3 μ m diameter.²⁸¹ Cooper and his colleagues made aligned and random electro-spinning CS/PCL nano/microfibers having diameters of 1100 nm, 400 nm, and 200 nm. Human ES cells (BG01v) were cultivated on several types of aligned and random nano/microfiber as well as on CS/PCL film and on collagen film in expansion media. They investigated neural (ectoderm; neurofilament and nestin), hepatic (endoderm; α -fetoprotein [ALP] and albumin), and osteogenic (mesoderm; ALP and Runx2), marker proteins and genes after one week of cultivation.⁸³ Microfibers with 1100 nm diameter facilitated hES cells to differentiate into neural cells, whereas nanofibers with 400 and 200 nm diameter promoted hES cells for the induction into hepatic and osteogenic cells (Type E protocol in Figure 4.3).⁸³ The alignment of nano/microfibers did not extensively give effects on the induction direction of hES cells in this research.

Xu and his colleagues investigated aligned PCL-gelatin nanofibers (mean diameter 270 nm) entrapped with up to 0.3 wt% RA (retinoic acid), which were formed as scaffold for hMS cells induced into neuronal lineages.²⁸⁸ These nanofibers, which were entrapped RA, released RA for at least 2 weeks. Human MS cells cultivated on aligned PCL-gelatin nanofibers without and with RA entrapment enhanced expression of neural markers; RIP (mature oligodendrocyte), GalC (oligodendrocyte marker), MAP2 (mature neuronal marker), and Tuj-1 (neuronal marker) (Table 4.1) at the protein and gene expression levels comparing with hMS cells cultivated on randomly oriented PCL-gelatin nanofibers or on conventional TCP dishes.²⁸⁸ Especially hMS cells cultivated on aligned PCL-gelatin nanofibers with entrapped RA exhibited extensively increased expression of neural markers in comparison to hMS cells on randomly oriented PCL-gelatin nanofibers with encapsulated RA or on aligned PCL-gelatin nanofibers without RA encapsulation.²⁸⁸ In particular, hMS cells cultivated on aligned PCL-gelatin nanofiber with entrapped RA, which generated the controlled release of RA with lower loading amounts (>8 times lower), increased RIP and MAP2 expression in comparison to hMS cells cultivated on nanofibers without RA entrapment in cultivation medium including high RA amount.²⁸⁸ Higher expression of MAP2 (mature neuronal marker) in hMS cells cultivated on aligned PCL-gelatin nanofiber with entrapped RA in comparison to the expression of glial markers at the protein and gene expression levels indicated that the nanofibers promoted neuronal induction of hMS cells. Moreover, positive

staining for synaptophysin was observed only in cells cultivated on aligned PCL–gelatin nanofiber with entrapped RA.²⁸⁸ This evidence indicates the advantage of the nanofiber-based biomaterials in promoting the neuronal induction of hMS cells and suggests the importance of the drug delivery method in guiding stem cell fate. Such biomimicking drug-encapsulating nanofibers (used as scaffolds) may suggest guiding cues to regulate the fate of recruited stem cells and would permit subsequent direct cell transplantation.

Dang and Leong studied aligned nanofiber matrices consisted of a thermally responsive hydroxybutyl CS (HBCS) blended without and with collagen type I.²⁷² Cell sheets were prepared by cooling hMS cells cultivated on the matrices to 4 °C, which allow the cells in the polymer-free cell sheets to hold their cytoskeletal alignment and elongated cell morphologies. The gene expression profiles of hMS cell differentiation lineages were examined on hMS cells in the aligned hMS cell sheets, where hMS cells were cultivated on aligned HBCS fiber matrices without and with collagen type I in expansion medium and not in differentiation induction medium. These lineages included myogenic, chondrogenic, and osteogenic induction.

Genes from all three differentiation lineages was expressed on hMS cells cultivated on both aligned HBCS/collagen or HBCS nanofiber matrices. It should be noted that a definitive up-regulation of myogenic genes was clearly observed on hMS cells cultivated on the aligned nanofiber matrices, which were compared with the genes expressed by hMS cells cultivated on TCP dishes and HBCS films. The elevated level of myogenin, a gene involved in muscle induction, which is the downstream of MyoD expression, indicated myogenic commitment, although MyoD expression was not observed in hMS cells on aligned nanofiber matrices.²⁷² The aligned nanofibrous morphology generated an elongated nuclear shape, and this elongated nuclear shape was regarded as a key factor in the myogenic induction of hMS cells. The aligned nanofiber presents topographical cue to induce cell alignment, actively regulating gene expression and influencing stem cell fate of induction.

Tendon is a connective tissue consisted of parallel collagen fibers. Human tendon stem/progenitor (hTSP) cells are known to exist within niches, which are made of primarily parallel collagen fibers and the niche plays a key function in controlling their differentiation and function.^{273,299–301} Polymeric and ECM electrospinning fibers may be suitable matrices to directly replicate the natural tendon ECMs. Then, Yin and his colleagues prepared randomly oriented and aligned PLLA fiber matrices, cultivated hTSP cells on the matrices, and studied the controlling of hTSP cell orientation and induction into tendons using the aligned electrospinning nanofiber.

Human TSP (tendon stem/progenitor) cells showed spindle-shaped shapes and were well-oriented on the aligned nanofiber matrices. The tendon-derived genes (scleraxis and Eya 2) was extensively expressed in hTSP cells cultivated on aligned nanofibers in comparison to the genes on randomly oriented nanofiber matrices in expansion medium and also in osteogenic induction medium (This is because of osteogenesis and tenogenesis sharing

typical signaling pathways).³⁰² Furthermore, alizarin red staining (mineralization) and ALP activity indicate that hTSP cells on randomly oriented nanofiber scaffolds went through induced osteogenesis, whereas hTSP cells on aligned nanofiber matrices suggested restriction of osteogenic induction.

Human TSP cells cultivated on aligned nanofiber matrices were transplanted into immunocompromised mice subcutaneously. The efficacy of inoculating hTSP cells on aligned nanofiber matrices in inducing tendon tissue regeneration was studied *in vivo*. It was found that aligned nanofibers contributed to induce the generation of tendon-like tissue cells and spindle-shaped cells from the evaluation of Masson's trichrome staining and H&E (hematoxylin and eosin) staining. These results indicate that aligned electrospinning nanofiber matrices have a controlling microenvironment for hTSP cell induction into tendon-like tissue, which might contribute to the establishment of desirable and intelligently engineered tendon in future.

4.3.3.2.3 Stem Cell Induction on Hybrid Nanofiber. Bone is made of highly organized nanofibrillar proteins (extensively composing of collagen type I), which contribute to patterns for the formation of crystalline calcium phosphate mineral in the form of HA (hydroxyapatite).^{267,303} A combination of nanofiber inorganic and organic composite matrices, such as (a) composite nanofiber matrices with biodegradable polymer and nano-sized demineralized bone powders²⁶⁴ and (b) calcium phosphates with nanofiber matrices^{267,268} would have ability for bone regeneration application. Seyedjafari and his colleagues generated electrospinning PLLA nanofiber coated with nano-hydroxyapatite (n-HA). They studied the ability of these spinning matrices for bone generation *in vitro* using human cord blood-derived unrestricted somatic stem (hCB-USS) cells under osteogenic induction conditions.²⁶⁷ Nanofibers coated with n-HA (n-HA/PLLA) facilitated proliferation, spreading, and attachment of hCB-USS cells. Higher ALP activity (an early marker of osteogenesis), bone-related gene (Runx2, osteonectin, osteocalcin) expression and biomineralization were found on nanofibers coated with n-HA in comparison to PLLA scaffolds with no n-HA coating.²⁶⁷ Moreover, the expression intensity of these markers were found to be higher in hCB-USS cells on PLLA nanofiber than in hCB-USS cells on TCP dishes. Furthermore, nanofiber matrices coated with n-HA displayed the ability for ectopic bone formation without exogenous cells *in vivo* after subcutaneous transplantation of the nanofiber matrices into mice.²⁶⁷

Chen and his colleagues also made nanocomposite matrices of n-HA dispersed in PCL using the electrospinning method.²⁶⁸ Osteogenic induction of MS cells was increased on the composite nanofiber matrices with an enhancement in n-HA content around 50%.²⁶⁸ The extent of mineralization was extensively higher in nanocomposite matrices with 50% n-HA that have Ca/P ratio similar to bone.

Nanofiber inorganic and organic composite matrices including PLLA and DBP (demineralized bone powders) were prepared using the electrospinning

method by Ko and his colleagues.²⁶⁴ PLLA and PLLA/DBP scaffolds were implanted into full-thickness bone defects generated in the central part of the rat cranial bones having 8 mm diameter. The results showed that a larger amount of newly generated bone extended across the defect area 3 months after transplantation with PLLA/DBP scaffold than in rats with no implant and in PLLA scaffold and that the defect size became almost 90% smaller.²⁶⁹ PLLA/DBP composite nanofiber matrices might be used as desirable matrices for the tissue engineering of bone tissue. The defect size reduced to 10% of its original defect size when PLLA/DBP composite nanofiber matrices were implanted.²⁶⁹

Nanofiber inorganic and organic composite matrices were found to be adequate for regulating MS cells into osteoblasts and for creating the mineralization of MS cells targeting for bone regeneration.

Fibrocartilaginous tissue such as the meniscus have critical load-bearing functions and rely on arrays of collagen fibers to hold tensile loads encountered during normal activities. The tissue structure is typically injured and have restricted healing capacity. Then, there is a demand for tissue-engineered replacement.²⁷¹ Baker and his colleagues studied scaffolds consisted of aligned nanofibers, which guided bovine MS cell orientation and the generation of organized ECMs under mechanical stimulation with the aim of regenerating the structural properties of these anisotropic tissue *in vitro*.²⁷¹ They investigated the effect of cyclic tensile loading on MS cell-laden nanofiber PCL matrices fabricated using the electrospinning technique.²⁷¹ MS cell fibrous gene expression (lysyl oxidase, fibronectin, and collagen type I) and collagen accumulation enhanced with mechanical stimulation, and the tensile moduli also enhanced by 15% relative to controls.²⁷¹ These results indicate that dynamic tensile loading promotes the maturation of MS cell-laden aligned nanofiber constructs, showing that recapitulation of the mechanical and structural niche of load-bearing tissues contributes to enhancement in functional properties, which may be exploited for regenerative medicine application.²⁷¹

4.3.3.3 Stem Cell Induction on Nanofibers Prepared Using Phase Separation

The phase separation is a typical method to create porous membranes with pore diameters ranging from 1–2 nm to 5–15 μm . The porosity of membranes with pore diameters on the order of nanometers is extensively low under common conditions (*e.g.*, less than 10%), indicating the membranes to be not appropriate for use of cell cultivation matrices. Some investigators have prepared nanofiber matrices rather than porous membranes using phase separation methods. These matrices should be valuable for the cultivation and induction of stem cells, however there have been only some articles relating stem cell cultivation and differentiation on nanofibers generated using phase separation methods in comparison to the nanofibers formed using the electrospinning method or using self-assembly protocol.

This is explained as nanofiber matrices generated using the phase separation method are extensively similar to the 2D morphologies of nanofiber mats, and stem cells cannot diffuse inside the nanofiber mats (scaffolds) and must hold on the biomaterial surface.

Nanofiber scaffolds are typically made from synthetic polymers as follows: (1) synthetic polymer is dissolved in a “good” solvent; (2) the polymer solution is cast on dishes or plates and phase-separated; (3) the polymer solution (gel) is immersed in solvent (water), and the solvent is removed from the gel, creating nanofiber mats (scaffolds); and (4) the nanofiber scaffolds are washed and then freeze-dried.

Smith and his colleagues created nanofiber scaffolds utilizing PLLA with the phase separation protocol to mimic the morphologies of natural ECMs. Their aim was to investigate the contribution of ECM morphologies to the induction of murine ES cells because natural ECMs, such as collagen type I, generally have nanofiber structure.³⁰⁴ The resulting nanofiber scaffolds had an average fiber diameter of 150 nm and a porosity of 93%. ES cells cultivated on the nanofiber scaffolds showed more extended morphology than ES cells on films made from the same PLLA or ES cells on gelatin-coated control plates. Moreover, ES cells cultivated on nanofiber scaffolds showed higher Brachyury expression, suggesting mesoderm induction, and had higher expression of osteogenic genes (mesoderm), such as bone sialoprotein, osteocalcin, Runx2, and collagen type I. However, the cells showed less expression of TUJ-1 (a neuronal marker, ectoderm) and nestin (a neural marker, ectoderm) than ES cells cultivated on gelatin-coated dishes or PLLA films (control experiments). They found that osteogenic induction was more highly facilitated when ES cells were cultivated on the nanofiber scaffolds than on gelatin-coated dishes or films. The mechanism of the high osteogenic induction evaluated on the nanofiber scaffolds might be elucidated by high sorption of fibronectin and/or serum proteins on the nanofibers made from PLLA. Some integrin subunits relating to cellular attachment to fibronectin ($\alpha 5 \beta 1$) and collagen type I ($\alpha 2 \beta 1$) were enhanced on the nanofiber scaffolds in comparison to the films.³⁰⁴ The enhancement in $\beta 1$ integrin transcription in ES cells on nanofiber scaffolds in comparison to those in ES cells on gelatin-coated dishes or films supports enhancement of mesoderm induction because enhanced $\beta 1$ integrin on stem cells is directly related to enhanced mesoderm induction while restricting neural induction.³⁰⁵ Enhanced fibronectin sorption on the nanofiber scaffolds in comparison to the gelatin-coated dishes and films likely promotes ES cell induction to the mesoderm and osteogenic lineages, as examined by $\alpha 5$ blocking assays.²²⁵ The nanofiber scaffolds have higher surface areas than conventional flat cultivation plates or films, which are preferable for the high sorption of ECM and/or serum proteins. High, somewhat desired sorption of ECM and serum proteins on nanofiber scaffolds made from selected chemical morphologies would be valuable in design of scaffolds and plates, which are adequate for stem cell induction into desired lineages.

Polyhydroxyalkanoates (PHA) such as PHB4HB (3-hydroxybutyrate and 4-hydroxybutyrate copolymer), PHBHHx (3-hydroxybutyrate and 4-hydroxyhexanoate copolymer), P4HB (3-hydroxybutyrate and 4-hydroxybutyrate copolymer), and PHB [poly(3-hydroxybutyrate)] are known to possess no cytotoxicity and excellent biodegradability *in vivo* and *in vitro*.^{306,307} Xu and his colleagues made nanofiber scaffolds using PHA, which is structurally similar to natural ECMs.³⁰⁶ Rat NS cells were cultivated on PHA nanofiber films and scaffolds. The viability of NS cells on PHA nanofiber scaffolds was extensively higher than the viability of NS cells on PHA films.³⁰⁶ This result suggests that nanofiber scaffolds with a 3D nano-structure would be preferable for NS cells to sorb growth factors, ECM proteins, and nutrients. NS cells cultivated on PHBHHx nanofiber scaffolds showed higher expression of β -III tubulin (neuronal marker) than NS cells on PHA nanofiber scaffolds, except for PHA films or PHBHHx.³⁰⁶ NSCs on PHBHHx nanofiber scaffolds seems to be more adequate for NS cell adhesion, synaptogenesis and synaptic outgrowth than other PHA films and nanofiber matrices.³⁰⁶

Collagen type I in native tissue is composed of three collagen polypeptide chains, which generate a rope-like super helix configuration and assemble into nanofibers ranging in size from 50–500 nm.^{304,308} Collagen 3D matrices are not typically composed of nanofiber networks, but rather formed porous sponges or hydrogels. There are only a few articles dealing collagen nanofiber scaffolds, which are generated with the phase separation techniques for stem cell cultivation and differentiation.³⁰⁹

Orza and his colleagues made gold-coated collagen nanofiber scaffolds by a single-step reduction process using HAuCl_4 , a reduction agent (sodium borohydrate or sodium citrate), and collagen solution.³⁰⁹ These scaffolds were electrically conductive because of their gold coating, which had fiber width of 20–65 nm depending on the preparation processes. Gold-coated collagen fibers are likely to support their nanofiber assemblies as well as their native rope-like super helix configuration. It was determined that placental-derived MS cells showed accelerated neural induction and displayed more neural lineage morphologic properties when MS cells were cultivated on gold-coated nanofiber scaffolds in neural induction media.^{230,309} MS cells cultivated on the gold-coated nanofiber scaffolds responded within 24–48 h to neuronal induction media by forming cells having neuronal-like extensions and more neuronal-like properties when compared with the cells cultured on conventional TCP dishes.³⁰⁹ One day of electrical stimulation with a neuronal induction protocol further facilitated to acquire neural morphologies, and after 2 days, MS cells were extensively oriented in the same direction.³⁰⁹ Transmitting electrical stimulation to the MS cells seems to be powerful because of the electrically conductive characteristics of the gold-coated nanofiber scaffolds.

The gold-coated nanofiber scaffolds could induce MS cells into myocytes extensively when MS cells were cultivated in myocyte induction media.³⁰⁹ MS cells on gold-coated nanofiber scaffolds were extensively positive for early cardiac-specific homeobox protein (Nkx2.5), a

cardiac hormone, and atrial natriuretic peptide (ANP, cardiac marker), in contrast to MS cells on conventional TCP plates.³⁰⁹

The phase inversion process allows for the simple generation of nanofiber scaffolds in comparison to the electrospinning method as well as peptide amphiphile self-assembly method. However, it is hard for cells to diffuse into the inside of the nanofiber scaffolds made by the phase separation process, which causes cells to typically retain on the scaffold surface. Then, stem cell cultivation and differentiation on nanofiber scaffolds prepared by electrospinning or peptide amphiphile self-assembly method are considered to be more valuable than those generated by the phase separation protocol for clinical applications because of the difficulty of 3D cultivation on nanofiber scaffolds created by the phase separation process. However, scaffolds having micropores generated by the phase separation process, such as microporous sponges, are typically utilized in regenerative medicine and tissue engineering for the entrapment and immobilization of stem cells.

4.3.4 Effect of Electrical and Mechanical Forces of Biomaterials on Induction Fate of hPS Cells

The electrical and mechanical forces, which affect cell cultivation materials also regulate the induction fate of hPS cells. Table 4.12 shows some studies

Table 4.12 The effect of physical factors of materials (electrical stimulation and mechanical stretching on biomaterials and stiffness of biomaterials) on hES and hiPS cell induction.¹⁸ Adapted from ref. 18 with permission from the Royal Society of Chemistry.

hPSCs	Cell culture materials	Physical effect (type)	Cell type (%)	Ref. (year)
<i>Mechanical stretching and electrical stimulation on biomaterials</i>				
hESCs (CHA-hES6)	Gold nanoparticles coated with fibronectin	Electrical stimulation (Type E)	Osteoblasts	13 (2009)
hESCs (HES2)	Gelatin sponge scaffolds	Mechanical stretching (Type A)	Cardiomyocytes (cTnT ⁺ cells 45%)	15 (2014)
<i>Elasticity of biomaterials</i>				
hESCs (H9)	PLLA/PLGA/PCL scaffolds coated with Matrigel	Elasticity of biomaterials (Type A)	Three germ layer cells	34 (2011)
hEDS (H9)	Elastin-like hydrogels	Elasticity of biomaterials (Type B)	Cardiomyocytes	45 (2012)
hESCs (H1, H9, UCLA1-6)	Oxygen-treated PDMS dishes	Elasticity of biomaterials (Type B)	Cardiomyocytes (cTnT ⁺ cells 9%)	310 (2013)
hESCs (H9)	Matrigel or fibronectin coated polyacrylamide hydrogels	Elasticity of biomaterials (Type E)	Cardiomyocytes (cTnT ⁺ cells 52%)	91 (2014)

of the effects of electrical and mechanical forces in materials on hPS cell differentiation.^{13,15,310}

Mihic and his colleagues examined the effect of cyclic stretch of gelatin matrices (Gelfoam) on the induction of hES cells into cardiomyocyte using the Type A method described in Figure 4.3 (Figure 4.34).¹⁵ A gelatin matrices inoculated with hES cell-derived cardiomyocyte was uniaxially cycled between relaxed and stretched states using a custom-built stretching equipment where a non-contact electromagnetic force was generated to stainless steel clamps, which were fixed to both end of the gelatin matrix (Figure 4.34B).¹⁵ Cyclical stretching was made with a frequency of 1.25 Hz (75 cycles per min) and a displacement of about 12%, and continuous cycling with each “stretch” generated approximately 35% of the cycle duration. Stretched gel matrices were cultivated with hES cell-derived cardiomyocyte under continuous and cyclical stretching for 3 days.

Histological analysis indicated that a higher proportion of cardiac cTnT⁺ cells was observed in stretched gelatin matrices than in non-stretched gelatin matrices, and flow sorting suggested a greater ratio of cardiomyocytes (Figure 4.34C).¹⁵ Ultrastructural assessment exhibited that the cells in

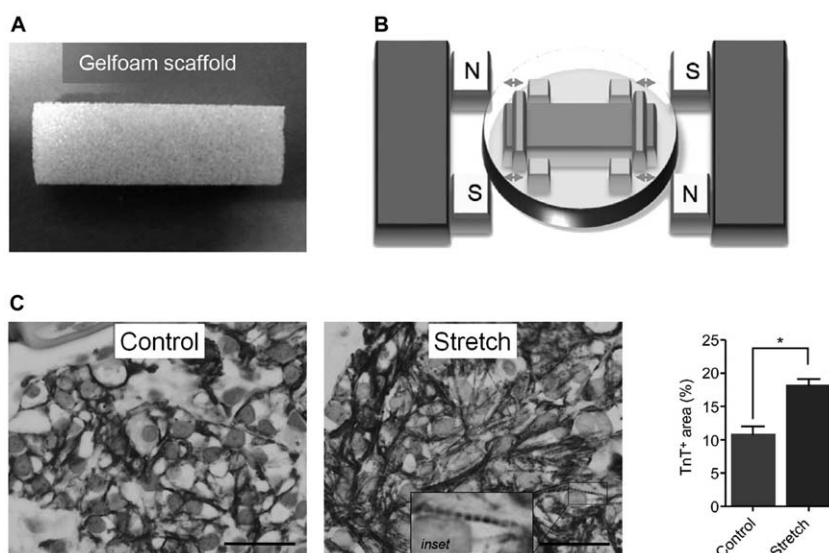


Figure 4.34 Stretched constructs generate a larger proportion of cardiomyocytes. (A) Gelfoam scaffold. (B) The stretching equipment generated non-contact electromagnetic forces, simultaneously displacing the construct at both ends. S=south pole and N=north pole of magnets. (C) Immunostaining for cTnT (TnT) showed higher expression in stretched constructs and well-defined striations with Z-banding running longitudinally along the stretched hES cell-derived cardiomyocytes (inset). Scale bar = 25 μ m.¹⁵

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stretched gelatin matrices carried a more mature phenotype, which was analyzed by a higher degree of better contractile elements, enhanced gap junction expression, and cell elongation. Real-time PCR showed an increased gene expression pattern, which was related to several genes relating cardiac ion channel and to cardiac maturation.¹⁵ Gelatin matrices with the size of 7×8 mm including hES cell-derived cardiomyocyte were surgically transplanted onto rat epicardial surface over damaged left ventricular free wall in an ischemia/reperfusion injury rat model (Figure 4.35).¹⁵ The epicardial transplantation of these construct onto ischemic rat heart indicated the feasibility of this method, which controlled to increased engraftment of the transplanted cells and survival in the stretched gelatin matrices (Figure 4.35D and E).¹⁵ This uniaxial stretching system might be considered as a platform for the creation of cardiac tissue-engineered matrices for regenerative medicine applications.

The development of electrically conductive biomaterials was considered for the potential use of such biomaterials in the medical field because of their potential to generate specific functions in applied tissues and cells *via* electrical stimulation.^{311,312} The effect of electrical stimulation on the induction of hES cells into targeted cell lineages were investigated by Woo and his colleagues (Type E method in Figure 4.3).¹³ In this research, hES cells (CHA-hES6) were cultivated on fibronectin-coated gold nanoparticles (diameter 20 nm); negatively ionized gold nanoparticles were coated onto a positively ionized PEI (polyethylene imine)-coated glass plate and fibronectin was subsequently coated onto the gold nanoparticle. Figure 4.36 describes a schematic picture of a fibronectin-coated gold nanoparticle surface on which hES cell induction was evaluated under alternative electrical stimulation.¹³ An Oct4 promoter-conjugated EGFP (green fluorescence protein) lentiviral vector was transduced on hES cells to examine their pluripotency. An evaluation of gene-modified hES cells that were adhered onto fibronectin-coated gold nanoparticles showed that the pluripotency marker (Oct4) was not observed after electrical stimulation.¹³

The expression of the mesoderm gene brachyury (T) was observed to be up-regulated by electrical stimulation, indicating that electrical stimulation promotes hES cells to induce into a mesodermal lineage, whereas the expression of the keratin 15 gene in the ectoderm and of the amylase gene in the endoderm, which were induced by electrical stimulation, were not observed when induced by non-electrical stimulation.¹³ The cartilage-associated proteins were expressed by hES cells under electrical stimulation, but collagen type II (chondrocyte differentiation) was not detected. Furthermore, the expression of the Runx2 and collagen type I osteogenic proteins and genes, which were generated by hES cells under electrical stimulation were detected to be higher than in the cells that were not induced by any electrical stimuli.¹³ These results indicate that electrical stimulation directly guides osteogenic induction in hES cells.

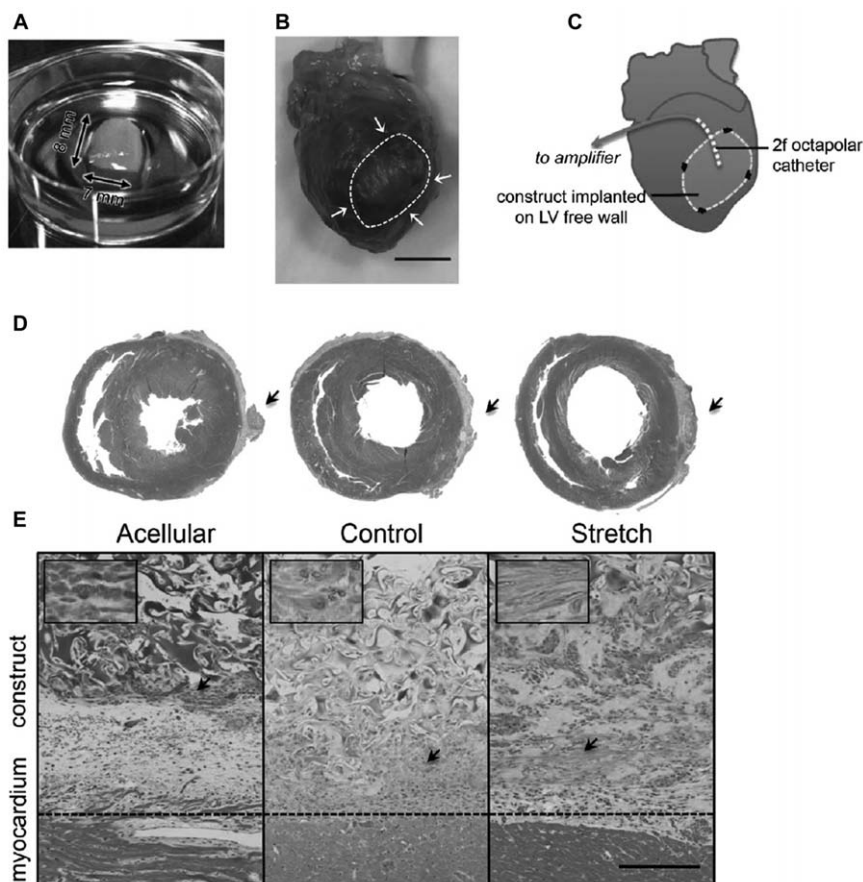


Figure 4.35 Characterization of hES cell-derived cardiomyocyte-seeded constructs transplanted in epicardium in rat cardiac I/R (ischemia/reperfusion) models. (A) Constructs (7×8 mm) were surgically transplanted onto the rat epicardial surface over an injured left ventricular free wall 1 week post-I/R. (B) Gross morphologies of an excised rat heart 2 weeks post-transplantation. The construct was clearly visible (outlined by the dashed line). Arrows show the positions of sutures (scale bar = 5 mm). (C) Schematic illustration of the orientation of the 2f octapolar catheter that was used to make direct open-chest ECG recordings simultaneously from the construct surface (distal electrodes) and the native myocardium (proximal electrodes). (D) Masson's trichrome staining of explanted heart cross-sections. Arrows show location of construct. (E) Masson's trichrome-stained epicardial-construct interfaces showed that stretched constructs exhibited better cell dispersion (both near the epicardium and up to at least 500 μ m away), underwent more Gelfoam remodeling, and contained more elongated cardiomyocytes (inset). The dashed line displays the approximate location of the epicardial border (scale bar 200 μ m).¹⁵

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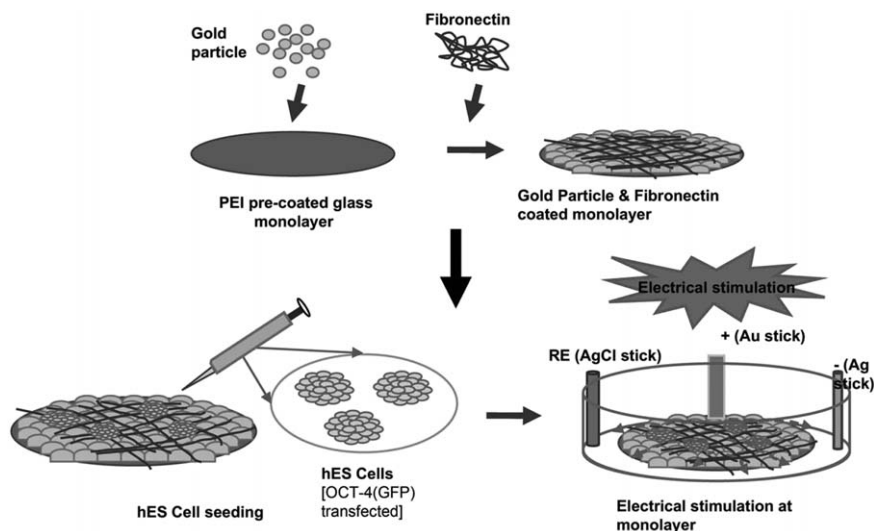


Figure 4.36 Schematic illustration of a fibronectin-coated gold nanoparticle for hES cell induction by alternative electrical stimulation.¹³ Reproduced from ref. 13 with permission from Elsevier, Copyright 2009.

4.4 Conclusions and Perspectives

The diverse differentiation potential of hES and hiPS cells generates difficulty in regulating induction toward an extensively desired cell lineage, which would be used in regenerative medicine.

The stiffness of materials in stem cell cultivation can control cell adherence and subsequently cell shape, cell phenotype, and FAs, especially in 2D cultivation. The differentiation of hES and hiPS cells is sensitive to biomaterial stiffness, especially during early stages of induction. The optimal choice of cell cultivation materials of adequate stiffness can enhance the efficiency of hES and hiPS cell induction into desired cell lineages, such as β cells, cardiomyocytes, dopamine-secreting cells, and retinal pigment epithelium.

A micro/nanograted (groove/ridge) patterned biomaterial of a specific width can facilitate the induction of hES and hiPS cells into β cells, cardiomyocytes, dopamine-secreting cells, and retinal pigment epithelium. Nanofibers hold a sophisticated 3D system for cultivation of hES and hiPS cells on nanopatterned biomaterials. Both electrical and mechanical stimulation of materials can contribute to enhance hPS cell induction into desired cell lineages.

The control of stem cell induction into desired lineages remains still not completely to be known. Cell cultivation biomaterials should be created with biomechanical, biochemical, and biophysical cues for this object. The development of materials needs multidisciplinary processes, and it will open the avenue to the controlled induction of stem cells into desired lineages.

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CHAPTER 5

Biomaterial Control of Differentiation of Human Embryonic Stem Cells and Induced Pluripotent Stem Cells

5.1 Introduction

We are facing a shortage of organs and tissues for patients who suffer loss and/or damage of their organs and tissues. Pluripotent stem cells, such as induced pluripotent stem (iPS) cells and embryonic stem (ES) cells are a valuable cell source for reconstructing and/or regenerating injured organs and tissues,^{1–5} which own higher differentiation properties and more pluripotency in comparison to adult stem cells (*e.g.*, adipose-derived stem cells and bone marrow-derived mesenchymal stem cells). The iPS cells hold similar characteristics to ES cells, and iPS cells were created by reprogram of somatic cells by transducing pluripotency genes.^{6–8} At present, iPS cells are also able to be prepared by reprogram of somatic cells using epigenetic-modifying small molecules or with pluripotency-related proteins.^{9–12} Pluripotent stem cells generated from iPS and ES cells have the potential to induce into several cell kinds, which are derived from the three germ layers: ectoderm cells (epidermal cell, retinal pigment epithelium, dendrocyte, astrocyte, and neuron), mesoderm cells (blood cell, cardiomyocyte, chondrocyte, and osteoblast), and endoderm cells (lung cell, hepatocyte, β cell, and hepatocyte). However, it is challenging to control pluripotent stem cells, especially hPS cells, inducing into targeted cell lineages because of their variety of induction ability of differentiation.

The stem cell induction is regulated by some independent factors in the hPS cell microenvironment: (1) cell-material interactions in cell cultivation; (2) physical factors, such as oxygen concentration, shear stress, and the stiffness of the cell cultivation materials; (3) cell-cell interactions, such as in co-cultivation; and (4) bioactive molecules, such as vitamins, cytokines, growth factors, and small molecules (Figure 5.1).³ An excellent strategy is to mimic the stem cell microenvironment for the induction of hPS cells into desired cell lineages using appropriate materials for hPS cell cultivation. The protocol for induction of hPS cells is more complicated because of the high differentiation potential and high pluripotency of hPS cells as well as different cultivation protocols for hPS cells in comparison to human adult stem cells, although human adult stem cells, such as amniotic fluid stem cells, adipose-derived stem cells, dental pulp stem cells, and bone marrow-derived stem cells can be differentiated using simple protocols, such as stem cell culture on materials in differentiation media. Typically, hPS cells are cultivated (a) on Matrigel-coating dishes, (b) on feeder cells, such as MEF (mouse embryonic fibroblasts, feeder layer), or (c) on appropriate materials to keep their pluripotency;^{3,13} while human adult stem cells are able to be cultivated on typical TCP (tissue culture polystyrene) plates. Subsequently, the hPS cell induction method is extensively different from the method for human adult stem cells.

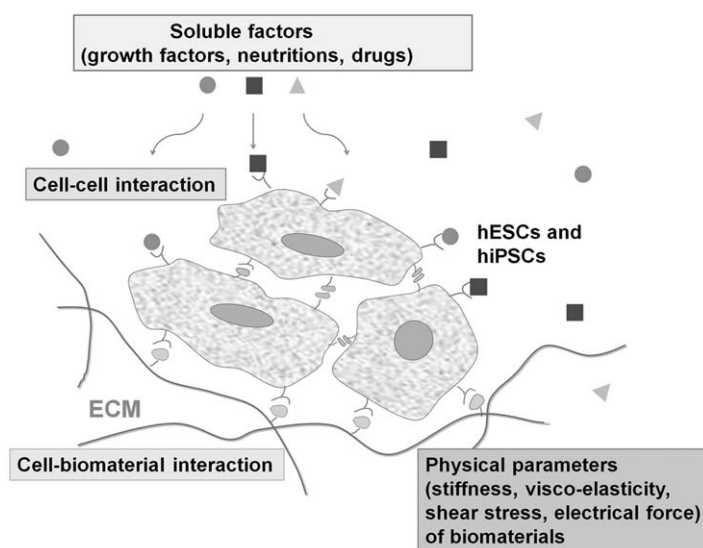


Figure 5.1 Illustration of the stem cell microenvironment. Stem cells are controlled by the following elements: (a) soluble active biomolecules, such as nutrients, growth factors, cytokines, and inhibitors, (b) cell-cell interactions, and (c) material-cell interactions. Physical properties of materials (d) also control stem cell fate.³ Adapted from ref. 3 with permission from the American Chemical Society, Copyright 2011.

Some outstanding original articles have been reported, which have focused on the protocols for inducing hPS cells into desired cell lineages, such as pancreatic cells, neurons, hepatocytes, retinal pigment epithelium, and cardiomyocytes.^{14–18} Unfortunately, there has been no systematic discussion, which focuses on the induction of hPS cells into desired cell lineages upon cultivation on different materials to the best of our knowledge. Then, this chapter describes several protocols for inducing hPS cells cultivated on materials and considers the appropriate materials for hPS cell induction into targeted cell lineages. Table 5.1 shows the proteins and genes used to examine the induction of stem cells into the three germ layers (ectoderm, mesoderm, and endoderm).

Table 5.1 Proteins and genes to analyze the induction of hPS cells into desired lineages (endoderm).⁵ Adapted from ref. 5 with permission from Elsevier, Copyright 2016.

Lineages	Cell type	Gene or proteins
Endoderm	Early endoderm	GATA4
	Early endoderm cells	Sox17 (sex determining region Y-box 17)
	Definitive endoderm	FOXA2
	Definitive endoderm	CXCR4
	Visceral endoderm	SOX7
	Primitive endoderm	E-cadherin (CDH1)
	Primitive endoderm	N-cadherin (CDH2)
	Endoderm cells	CER
	Endoderm cells	GATA6
	Endoderm cells	Ihh
	Endoderm cells	Amylase
	Endoderm cells	MIXL1 (mix-like homeobox protein 1)
	Endoderm cells	GSC (goosecoid homeobox)
	Endoderm cells	FOXQ1
	Endoderm cells	FOXA2 (forkhead box A2)
	Visceral endoderm cells	AFP (α -fetoprotein)
	Primitive gut tube	HNF1 β
	Islet-like cells, beta cells	PDX1
	Islet-like cells, beta cells	c-peptide
	Islet-like cells, beta cells	PC2
	Islet-like cells, beta cells	NKX6.1
	Islet-like cells, beta cells	Glucokinase
	Islet-like cells, beta cells	GLUT2
	Type I pneumocyte	Aquaporin-5 (AQ5)
	Type I pneumocyte	Caveolon-1
	Type I pneumocyte	T1 α
	Type II pneumocyte	Surfactant protein A (SPA)
	Type II pneumocyte	Surfactant protein B (SPB)
	Type II pneumocyte	Surfactant protein C (SPC)
	Hepatocytes	ASAGPR (asialoglycoprotein receptor)
	Hepatocytes	AFP (alpha fetoprotein)
	Hepatocytes	ALB (albumin)
	Hepatocytes	CK18 (cytokeratin 18)
	Hepatocytes	HNF4a (hepatocyte nuclear factor 4 α)
	Hepatocytes	TO (tryptophan oxygenase)
	Hepatocytes	G6P (glucose-6-phosphate)

Table 5.1 (Continued)

Lineages	Cell type	Gene or proteins
Mesoderm	Hepatocytes	GSTA1, GSTA2
	Hepatocytes	CYP1A2, CYP2C9, CYP3A4, CYP3A5, CYP3A7, CYP7A1
	Hepatocytes	UGT1A1
	Hepatocytes	Serpina1 (serine peptidase inhibitor a1)
	Hepatocytes	Ttr
	Early mesoderm cells (mesendoderm)	Brachyury (T)
	Early mesoderm cells	EOMES
	Early mesoderm cells	FLK1
	Early mesoderm cells	Goosecoid
	Early mesoderm cells	MIXL1
	Early mesoderm cells	WNT3
	Early mesoderm cells	PITX1
	Early mesoderm cells	GSC
	Mesoderm cells	BMP4
	Mesoderm cells	FOXF1 (forkhead box F1)
	Mesoderm cells	MEOX1 (mesenchyme homeobox 1)
	Mesoderm cells	KDR (kinase insert domain receptor, Flk1)
	Mesoderm cells	KDR (Flk1)
	Mesoderm cells	CXCR4
	Mesoderm cells	PDGFRA, PDGFRB
	Mesoderm cells	GATA1, GATA2
	Mesoderm cells	TBX4
	Undifferentiated mesenchymal stem cells	CD105
	Mesoderm cells	TBX5
	Early osteoblasts	RUNX2
	Late osteoblasts	Osteocalcin (BGLAP)
	Late osteoblasts	Osteopontin (SPP1)
	Hemato-endothelial cells	CD34
	Hematopoietic progenitor cells	CD34
	Hematopoietic progenitor cells	cKit + sca-1 +
	Early hematopoietic cells	RUNX1
	Hematopoietic cells	CD133
	Hematopoietic cells	Cav1
	Hematopoietic cells	CD41
	Hematopoietic cells	SCL
	Early endothelial cells	TIE2
	Endothelial cells	CD144
	Endothelial cells	CD31
	Endothelial cells	DCN
	Endothelial cells	N-cadherin
	Endothelial cells	SOX17
	Endothelial cells	IGFBP4
	Endothelial cells	WNT3a
	Endothelial cells	vWF-8 (von Willebrand factor-8)
	Endothelial cells	PECAM
	Vascular (endothelial) cells	VE-cadherin

Table 5.1 (Continued)

Lineages	Cell type	Gene or proteins
Ectoderm	Early ectoderm	NCAM1 (Neural cell adhesion molecule 1)
	Early ectoderm	BLBP (Brain lipid binding protein)
	Ectoderm cells (neural precursors)	SOX1 (Sex determining region Y-box 1)
	Ectoderm cells	ZIC1 (Zic family member 1)
	Ectoderm cells	FGF5
	Ectoderm cells	Keratin 15
	Neural progenitor cells	PSA-NCAM
	Neural progenitor cells	PAX6
	Neural progenitor cells	Nestin
	Neural progenitor cells	NGN1
	Neural progenitor cells	BLBP (Brain lipid binding protein)
	Neural crest stem cells	AP2
	Neural crest stem cells	SOX10
	Neural crest stem cells	BRN3a
	Neural crest stem cells	Slug
	Neural crest stem cells	ErbB3
	Neural crest stem cells	Vimentin
	Neural crest stem cells	HNK1
	Neural crest stem cells	p75
	Neural crest stem cells	Integrin- α 4 (CD49d)
	Immature neuron	β III tubulin (Tuj1), TUBB3
	Forebrain neural cells	FORSE-1
	Forebrain neural cells	BF-1
	Forebrain neural cells	FOXP1
	Midbrain neural cells	OTX2
	Midbrain neural cells	EN1 (Engrailed 1)
	Midbrain neural cells	LMX1a, LMX1b
	Midbrain neural cells	PITX3
	Midbrain neural cells	NURR1
	Midbrain neural cells	FOXA2 (forkhead box protein A2)
	Midbrain neural cells	GIRK2 (G protein-activated inward rectifier potassium channel), KCNJ6
	Hindbrain/spinal cord cells	HOXB4
	Neuron	NeuroD1
	Neuron	GABA
	Neuron	BRN3a
	Neuron	Peripherin
	Neuron	HuC/D (human neuronal protein)
	Neuron	MAP2 (microtubule-associated protein 2)
	Neuron	MBP (Myelin Basic Protein)
	Neuron	NEF3 (Neurofilament 3)
	Neuron	NF (Neurofilament)
	Neuron	Synaptophysin
	Neuron	NeuroN
	Neuron	Engrailed-1
	Dopaminergic neurons	ALDH1a1
	Dopaminergic neurons	TH (Tyrosine hydroxylase)
	Dopaminergic neurons	LMX1a (LIM homeobox transcription factor 1 alpha)
	Dopaminergic neurons	FOXA2
	Dopaminergic neurons	En1

Table 5.1 (Continued)

Lineages	Cell type	Gene or proteins
	Dopaminergic neurons (Late stage)	Aadc NURR1
	Dopaminergic neurons (Late stage)	VMAT2 (vesicular monoamine transporter 2)
	Dopaminergic neurons (Late stage)	PITX3
	Dopaminergic neurons (Late stage)	SCN1a (voltage gated sodium channels)
	Dopaminergic neurons	Dat
	Motor neuron progenitors	OLIG2
	Motor neuron	HB9
	Motor neuron	ISL1
	Motor neuron	ChAT
	Astrocytes (glial cells)	GFAP (Glial fibrillary acidic protein)
	Schwann cells	S100 β
	Oligodendrocytes	O4
	Oligodendrocytes	RIP
	Oligodendrocytes	GalC
	Oligodendrocytes	MBP
	Oligodendrocytes	O1
	Retinal precursor cells	CRX
	Retinal precursor cells	BLIMP1
	Retinal precursor cells	OPSINSW (s-opsin)
	Retinal precursor cells	RCVRN (recoverin)
	Retinal pigment epithelium	MITF
	Retinal pigment epithelium	RPE65
	Retinal pigment epithelium	BEST, PMEL, PEDF, TYR
	Keratinocyte precursors	K14 (Keratin 14)
	Mesoderm cells	MIXL1
	Mesoderm cells	WNT3
	Mesoderm cells	T (Brachyury)
	Juvenile or prechondrogenic cells	Collagen type IIA
	Adult or fully differentiated chondrocytes	Collagen type IIB
	Chondrocytes	Sox9
	Chondrocytes	Collagen type II
	Osteochondrocytes	Collagen X
	Cardiac progenitor cells	Flk-1
	Cardiomyocytes	TBX5
	Cardiomyocytes	ANP (atrial natriuretic peptide)
	Cardiomyocytes	MHC (myosin heavy chain), α -MHC
	Cardiomyocytes	MLC2a (myosin light chain 2A), MYL7
	Cardiomyocytes	NKX2.5
	Cardiomyocytes	Troponin C, troponin I
	Cardiomyocytes	TNNT2
	Cardiomyocytes	MF20 (α MHC), sarcomeric myosin
	Cardiomyocytes	GATA4

Table 5.1 (Continued)

Lineages	Cell type	Gene or proteins
	Cardiomyocytes	TEF1
	Cardiomyocytes	MEF2C
	Cardiomyocytes	Gap junction protein connexin-43
	Cardiomyocytes	α -Cardiac actin
	Cardiomyocytes	Brain natriuretic protein
	Cardiomyocytes	ACTC
	Cardiomyocytes	cTnT (cardiac troponin T)
	Cardiomyocytes	CASQ2 (calsequestrin)
	Cardiomyocytes	SERCA2
	Cardiomyocytes	ISL1
	Cardiomyocytes	Actinin
	Cardiomyocytes	Connexin 43
	Cardiomyocytes	SIRPA (signal-regulatory protein alpha), CD172a
	Smooth muscle cells	α -SMA, SMA (smooth muscle actin)
	Smooth muscle cells	CNN1 (calponin)
	Smooth muscle cells	Caldesmon
	Smooth muscle cells	SM-myosin
	Smooth muscle cells	MyoCD (myocardin)
	Smooth muscle cells	SM22 α (TAGLN)
	Smooth muscle cells	SMMHC
	Smooth muscle cells	Smoothelin
	Skeletal myoblasts	NCAM
	Skeletal myoblasts	MYOG (myogenin)
	Skeletal myoblasts	PAX7
	Myoblasts	MYOD, MyoD
	Myoblasts	MHC (myosin heavy chain)
	Myoblasts	PAX3
	Myoblasts	MY5
	Myoblasts	MY6
	Myoblasts	Myogenin
	Adipocytes	PPAR- γ (peroxisome proliferator-activated receptors γ)
	Adipocytes	Adipsin
	Adipocytes	ADRP (PLIN2, adipophilin, adipose differentiation-related protein)
	Adipocytes	Leptin
	Adipocytes	GLUT4 (glucose transporter type 4)
	Adipocytes	aP2 (adipocyte protein 2)

5.2 Induction of hPS Cells into Neural Lineages

hPS cells can induce into some types of neural cells within the central nervous system, such as neural crest stem cells, oligodendrocytes, astrocytes, Schwann cells, glial cells, motor neurons, gamma-aminobutyric acid (GABA) inhibitory neurons, and dopaminergic (DA) neurons.¹⁹ Among these cells, GABA inhibitory neurons and dopaminergic neurons that undergo degeneration in epilepsy disease,²⁰ and Parkinson's disease,²¹ respectively, should be promising for cell therapies in regenerative medicine, if the

neurons can be extensively induced from hPS cells with enough cell numbers at GMP grade. In this session, we study the appropriate induction methods and biomaterial design for production of targeted neural lineages from the induction of hPS cells.

We can categorize different induction protocols of hPS cells into several kinds of typical categories. Major induction protocols of hPS cells into the desired lineages of cells are shown in Figure 5.2, which are extensively different protocols of induction of adult stem cells. There are three major neural induction protocols for hPS cells: (a) direct induction method on materials (Type E and G induction protocols),^{16,22–38} (b) induction through EB generation (Type A–D induction protocols in Figure 5.2),^{19,23,30,39–50} and (c) stromal-induced induction (SDIA, stromal cell-derived inducing activity, Type F induction protocol in Figure 5.2),^{33–36,45,47,51} We compare and discuss these methods in the following sections. Several studies of neural induction are described in Table 5.2,^{16,19,22–77} and their typical protocols are shown in Figure 5.3.

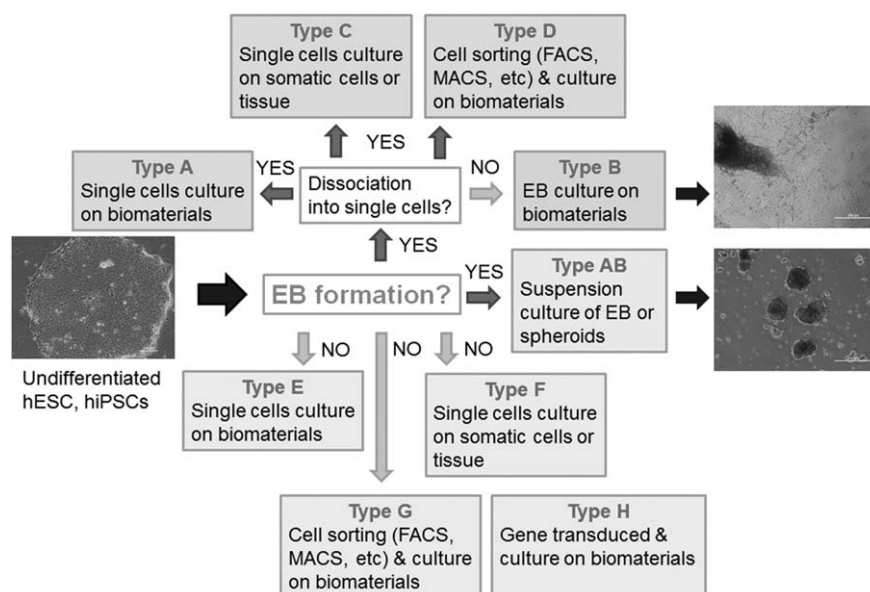


Figure 5.2 Protocols for inducing hPS cells into specific cell lineages. Differentiation of hPS cells is categorized based on EB generation (Type D, C, B, A, and AB induction protocols) or its absence (Type H, G, F, and E induction protocols). Aggregated or single cells are seeded on material in the Type E and A induction protocols. The cells are cultivated on somatic cells in the Type F and C induction protocols. Cell sorting using MACS or FACS and cell sorting based on morphologies are used in the Type G and D induction protocols. Cell selection is done after gene transfection or transduction in the Type H induction protocol.⁸⁷ Adapted from ref. 87 with permission from the Royal Society of Chemistry.

Table 5.2 Differentiation of hPS cells into neurons cultured on biomaterials.⁵ Adapted from ref. 5 with permission from Elsevier, Copyright 2016.^a

hPSCs	Cell culture materials	Methods	Cell type (%)	Ref. (year)
hiPSCs	Hyaluronan and methylcellulose hydrogels immobilized with RGD and PDGF	Type AB	Oligodendrocytes	61 (2016)
hESCs (H9)	PLGA/PLA scaffolds	Type A	Neural cells	40 (2005)
hiPSCs (SFS.1)	Matrigel-coated nanograting PDMS substrates	Type A and Type E	Neurons	30 (2013)
hiPSCs	Matrigel-coated plate	Type A	Retinal progenitor cells	77 (2017)
hESCs (HES-3)	LN-1 coated dishes	Type B	Neuroprogenitor cells	41 (2013)
hiPSCs (1-MCB-01)	PCL, retinoic acid encapsulated PCL	Type B	Neural progenitor cells	52 (2015)
hiPSCs (ADAFe4-iPS38-2)	CellStart-coated dishes	Type B and D	Neural crest stem cells	42 (2013)
hESCs (HES-3, H7), hiPSCs (iPS-IMR90)	Matrigel- and laminin-immobilized TCPS	Type B and E	Neural cells	23 (2013)
mESCs (D3)	Decellularized ECM derived from ESCs, EB, and neural progenitor cells	Type B	Neural cells	62 (2016)
hiPSCs (iPSK3)	Geltrex-coated plate	Type B	Neural cells	63 (2018)
hiPSCs	PLO/laminin-coated plate	Type B	Huntington's disease neural cell	64 (2017)
hESCs (H9)	Poly(acrylic acid) grafted carbon nanotube films	Type B	Neurons	43 (2009)
hESCs (H9)	LN and POL dishes and heparin-grafted PLLA nanofibers with EGF or FGF2 adsorption	Type B and Type D	Neurons	44 (2010)
hESCs (HSF6)	Polypyrrole coated with laminin oligopeptide (p20)	Type B and Type F	Neurons (β III-tubulin ⁺ cells, 25%)	45 (2010)
hESCs (SH42)	PDL/LN-coated dishes and electrospun silk fibroin nanofibers	Type B and D	Neuron (β III-tubulin ⁺ cells)	46 (2012)
hiPSCs	PCL-BSA-BDNF	Type B	Neurons	53 (2015)
hiPSCs (iPSK3)	Geltrex-coated plate	Type B	Neurons	65 (2016)
hESCs (H9)	PLO/LN-coated dishes	Type B	Motor neurons	54 (2015)
hiPSCs [iPS (foreskin)-1]	Fibrin hydrogels (3D)	Type B	Spinal neurons	66 (2017)

hESCs (I6)	CellStart-coated dishes, PO/LN-coated dishes	Type B and Type F	Dopaminergic neurons (TH ⁺ cells 25–35%), astrocytes, oligodendrocytes	47 (2009)
hiPSCs	Geltrex-coated TCPS	Type B	Dopaminergic neurons (TH ⁺ cells 2–5%)	48 (2012)
hESCs (H1)	Matrigel-coated dishes, PLO and LN-coated dishes	Type B and D	Dopaminergic (TH ⁺ cells, 60%) and GABAergic neurons (GABA ⁺ cells, 86%)	49 (2014)
hESCs (H7)	Matrigel-coated and PLL/LN-coated dishes	Type B	Oligodendroglia (GalC + 95%, RIP + 95%, O4 + 85%)	55 (2005)
hiPSCs (iPSK3, SY-UBH)	Geltrex-coated plates	Type B	Forebrain cortical organoids	67 (2018)
hESCs (WA09)	Matrigel-coated dishes	Type B and Type G	Photoreceptor precursor cells (CRX ⁺ , 93%)	56 (2013)
hESCs (H1)	PDL-Matrigel-coated dishes	Type B	Retinal cells	57 (2006)
hESCs (H1)	Microchannel PLGA coated with Matrigel	Type B	Retinal cells	58 (2012)
hESCs (H9)	Scaffolds composed of gelatin, chondroitin sulfate and hyaluronic acid	Type B	Retinal cells	68 (2018)
hESCs (H1, H9)	PLO/LN-coated dishes	Type D	Neural precursor cells (Nestin ⁺ cells 96%)	39 (2001)
hiPSCs, hESCs (H1, H9)	CellStart-coated dishes, PLCL and PPG nanofibrous tubular scaffolds with Matrigel	Type D	Neural crest stem cells, Schwann cells (AP2 ⁺ cells, 80–95%)	50 (2011)
hESCs (HES-3), hiPSCs (IMR90)	Laminin-1 coated plates	Type D	Dopaminergic neurons	19 (2012)
hESCs (HS181, HS360, H9), hiPSCs (C3, C5)	Recombinant spider silk protein (4RepCT) film containing IKVAV, RGD, YIGSR, PQVTRGDVFTM	Type E	Neuroectoderm cells, definitive endoderm cells, cardiomyocytes	59 (2014)
hESCs (HES-3), hiPSCs (IMR90)	Matrigel-coated cellulose DE-53 microcarriers	Type E	Neural progenitor cells (PSA-NCAM ⁺ cells, 60–84%), neurons, astrocytes, oligodendrocytes	22 (2013)

Table 5.2 (Continued)

hPSCs	Cell culture materials	Methods	Cell type (%)	Ref. (year)
Mouse ESCs (46C)	Fibrin hydrogels tethering $\alpha 6\beta 1$ integrin ligand [T1 (GTTWSQCSKS), HYD1 (KIKMVISWKG), A5G81 (AGQWHRVSVRWG)]	Type E	Neural stem/progenitors and neurons	86 (2017)
hiPSCs (iPSK3)	Decellularized ECM derived from ESCs, EB, and neural progenitor cells	Type E	Neural cells	69 (2015)
hiPSCs (iPSK3)	Polyurethane foam scaffolds	Type E	Neural cells	70 (2017)
hESCs (H1)	Matrigel-coated plates	Type E	Neural cells	71 (2018)
hESCs (H1)	Vitronectin-coated circular micropatterned island on PDMS surface	Type E	Neuroepithelial cells and neural plate border cells	72 (2018)
hESC (SA002)	Polyether-based polyurethane electrospun nanofibers	Type E	Neurons (β III-tubulin ⁺ 80% and TH ⁺ 80%)	24 (2009)
hESCs (BG01)	Gelatin-coated dishes	Type E	Neurons (TnTx cells, 79%)	25 (2009)
hESCs (WA-09), hiPSCs (iPS-14, iPS-27)	Matrigel-coated dishes	Type E	Neurons (PAX6 ⁺ , TH ⁺ , motor neuron cells)	26 (2009)
hESCs (HES1, HES2, H7)	In suspension and PDL/LN-coated dishes	Type E	Neurons (PSA-NCAM ⁺ cells, 91%)	27 (2010)
hESCs (H9)	Gelatin-coated polyurethane acrylate ridge/groove pattern array	Type E	Neurons	28 (2010)
hESCs (Royan H6)	Matrigel-coated Ultraweb (polyamide) electrospun nanofibers	Type E and Type G	Neurons (β III-tubulin ⁺ cells, 82%), (MAP2 ⁺ cells, 66%)	29 (2011)

hESCs (SA121)	Polyurethane electrospun nanofibers coated with PLO and LN	Type E	Neurons	31 (2014)
hESCs (H1)	POL/LN-coated nanograting PDMS	Type E	Neurons	60 (2015)
hESCs (H1)	PLO/LN-coated dishes	Type E	Neurons	60 (2015)
hiPSCs	Electrospun polystyrene nanofibers	Type E	Neurons	73 (2016)
hESCs (H1, H9), hiPSCs (2C6, SeV6)	Matrigel, PLO/LN/FN-coated dishes	Type E	Midbrain dopamine neurons (FOXA2 ⁺ /TH ⁺ cells, TH ⁺ cells, 75%)	16 (2011)
hiPSCs	LN521-coated dishes	Type E	Dopaminergic neurons	32 (2014)
hESCs (RC17, H9, Mshef7, HS980)	Laminin-111 coated plates	Type E	Dopaminergic progenitors	74 (2017)
hESCs (H1), hiPSCs	Matrigel-coated plates	Type E	GABAergic neuron	75 (2017)
hiPSCs	Matrigel-coated plates	Type E	Sensory neurons	76 (2018)
hESCs (H1, HES3)	PLO/LN-coated dishes	Type F and G	Neural precursors	33 (2005)
hESCs (H9, RUES1, I-8)	PLO/LN-coated dishes	Type F and G	Neural crest stem cells	34 (2007)
hESCs (H1, H9)	PO/LN-coated dishes	Type F and G	Dopaminergic neurons (TH ⁺ cells, 65–80% among β III-tubulin ⁺ cells)	35 (2004)
hESCs (H7, H9)	PO/LN-coated dishes	Type F and G	Dopaminergic (TH ⁺ cells, 24%), neurons	36 (2007)
hESCs (BG01)	PLL/LN-coated dishes	Type F	Dopaminergic neurons (TH ⁺ cells, 34%)	51 (2008)

^aBSA, bovine serum albumin; LN, laminin; PCL, polycaprolactone; PDL, poly-D-lysine; PDMS, polydimethylsiloxane; PLGA, poly(lactic-co-glycolic acid); PLL, poly-L-lysine; PLLA, poly(L-lactic acid); PLO, polyornithine; TCPS, tissue culture polystyrene.

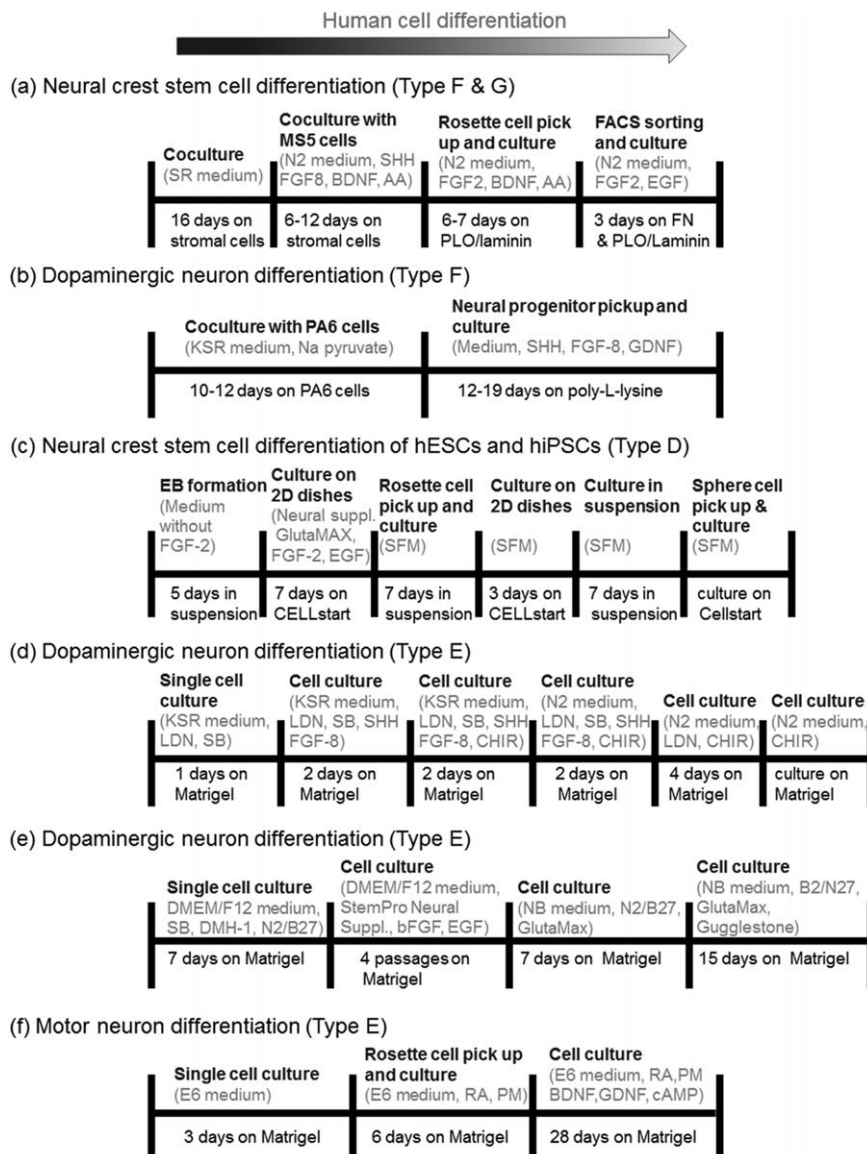


Figure 5.3 Timelines of protocols for hPS cell induction into cells of neural lineages. (a) Protocol for differentiation of hPS cells into neural crest stem cells using the Type F and G induction protocols studied by Lee and his colleagues.^{5,34} (b) Protocol for differentiation of hPS cells into dopaminergic neurons using the Type E induction protocol studied by Vazin and his colleagues.^{5,51} (c) Protocol for differentiation of hPS cells into neural crest stem cells using the Type D induction protocol studied by Wang and his colleagues.^{5,50} (d) Protocol for differentiation of hPS cells into dopaminergic neurons using the Type E induction protocol studied by Kriks and his colleagues.^{5,16} Adapted from ref. 5 with permission from Elsevier, Copyright 2016.

5.2.1 Stromal-induced Differentiation into Neural Lineages

hPS cell induction toward the neuroectoderm lineage can be made by co-cultivation of hPS cells with stromal cells, such as MS-5 and PA6 cells;^{34–36,47,51,78} this method is known as a Type F induction protocol. Lee and his colleagues generated neural crest cells from hES cells by co-cultivation with murine stromal MS-5 cells (Figure 5.3(a)).³⁴ Rosette morphologies were observed and chosen after 3–4 weeks of induction, and then, the cells from the rosette configurations were cultivated on polyornithine (PLO)/laminin (LN)-coated plates in media including N2. After 7 days, the cells at passage one were harvested and selected by FACS (fluorescence activated cell sorting) using markers of neural crest stem cells (HNK1 and p75, Table 5.1).³⁴ hES cell-derived neural crest stem cells prepared using this method were subsequently examined. The neural crest stem cells were shown to proliferate *in vitro* and were induced toward peripheral nervous system lineages (Schwann cell and peripheral neuron) and mesenchymal cells (chondrogenic cells, osteogenic cells, adipogenic cells, and smooth muscle cells).³⁴ From transplantation into adult mouse hosts and developing chick embryo, the neural crest stem cell exhibited differentiation, migration, and survival, which showed neural crest stem cell characteristics.³⁴

Vazin and his colleagues examined stromal cell-inducing potential in the induction of dopaminergic neurons from hES cells.⁵¹ hES cells were co-cultivated with PA6 (murine stromal) cells for approximately two weeks and subsequently induced with GDNF (glial cell-derived neurotrophic factor), FGF-8, and SHH (sonic hedgehog) (Figure 5.3(b)). Dopaminergic tyrosine hydroxylase expressing (TH⁺) cells with 35% were induced from hES cells after 18 days. When PA6 cells were fixed with paraformaldehyde or ethanol or PA6 cells were irradiated with ionizing radiation, the number of TH⁺ cells, which induced from hES cells, reduced by threefold, while PA6 cells that were treated with mitomycin-C caused a less reduction to 30% of induction.⁵¹ The neural-differentiating effects of hES cells were minimally effected by fixation of PA6 cells or mitomycin-C treatment, which were examined by β III tubulin expression of induced hES cells, but was reduced to 50% when PA6 cells after irradiation were utilized. Conditioned media from PA6 cells including heparin can guide hES cell induction into TH⁺ cells. However, the use of condition media was not so effective in comparison to the co-cultivation system with PA6 cells. Then, the findings indicated that the surface activity of PA6 cells is important for the neural induction of hES cells, regardless of whether PA6 cells are irradiated or fixed, but secreting molecules from PA6 cells are probably valuable for the induction of hES cells into TH⁺ neuron.⁵¹

The merit of stromal cell-driving differentiation of hPS cells into the neural lineages is the simple protocol of the induction, which requires only a few processes and few growth factors and/or small molecules in comparison to other methods, which do not use co-cultivation systems. On the other hand, the use of xeno biomaterials, such as murine stromal cells, restricts the use of such induced cells in clinical therapies.

5.2.2 Induction into Neural Lineages Through EB Generation

Animal-derived biomaterials, especially murine cells in co-cultivation, hamper the clinical use of the hPS cells induced into specific lineages. Currently, some neural inductive molecules, including SHH, Noggin, and FGF families, were used to differentiate hPS cells into desired neural cell lineages. SHH works an important function to specify distinct dorsal–ventral identity and neuronal cell fate.⁷⁹ Noggin is a signaling molecule playing a key role in facilitating somite patterning in the developing embryos.⁸⁰ Some methods for the induction of hPS cells into neural lineages through EB generation with the use of growth factors and small molecules have been proposed (Type A–D induction protocols).^{19,23,30,39–50} This is because EBs can induce into any kinds of somatic cells, which derives from the three germ layers.

Wu and his colleagues¹⁹ and Chan and his colleagues⁴¹ prepared neuroprogenitors from hES cells with no use of co-cultivated murine cells. They cultivated hPS cells on extracellular matrix (ECM)-coated dishes after EB generation (Type B and Type D induction protocols), which is similar to the protocol used by Wang and his colleagues (Figure 5.3(c)).⁵⁰ hES cells were cultivated with EB generation for 4 days on a non-adhesive plate. EBs were seeded on laminin (LN)-1-coated plates in media including Noggin, B27, and N2 and cultivated for 10 days. Then, compact clumps of rosettes (Figure 5.4) were observed and cultivated as neuroprogenitors (neurospheres) on a non-adhesive plate in media including FGF-2, EGF, B27, and N2 for 7 days.^{19,41} To induce differentiation into dopaminergic (TH⁺) neurons, neuroprogenitors were cultivated in media containing ascorbic acid, SHH, and FGF-8 on LN-1-coated plates. After 6–11 days, reformed rosettes were observed and cultivated in media including SHH, FGF-8, GDNF, BDNF, and cyclic adenosine monophosphate on LN-1-coated plates for 14 days to form dopaminergic (TH⁺) neuron. The percentage of dopaminergic (TH⁺) neurons formed using this method was found to be around 30% among all neurons (TH⁺ cell percentage among MAP2⁺ cells).¹⁹

Similar protocols have been developed for inducing hPS cells into several neural cell lineages including dopaminergic neurons.^{47–49}

5.2.3 Direct Induction into Neural Lineages on Materials with No EB Generation

Typical methods for inducing hPS cells into dopaminergic neurons need an EB generation stage and the sorting of cells having a rosette structure. On the other hand, the induction of hPS cells through EB generation may be a less specific protocol for production of specific cell lineages and produces a lower percentage of desired cells, such as GABAergic neuron or dopaminergic neuron (the percentage of TH⁺ cells is around 20–35%, Table 5.2). Then, dopaminergic neuron formed from hPS cells through EB generation worked poorly in animal models for Parkinson's disease.⁸¹ Some investigators have started to create more direct protocols for inducing hPS cells into

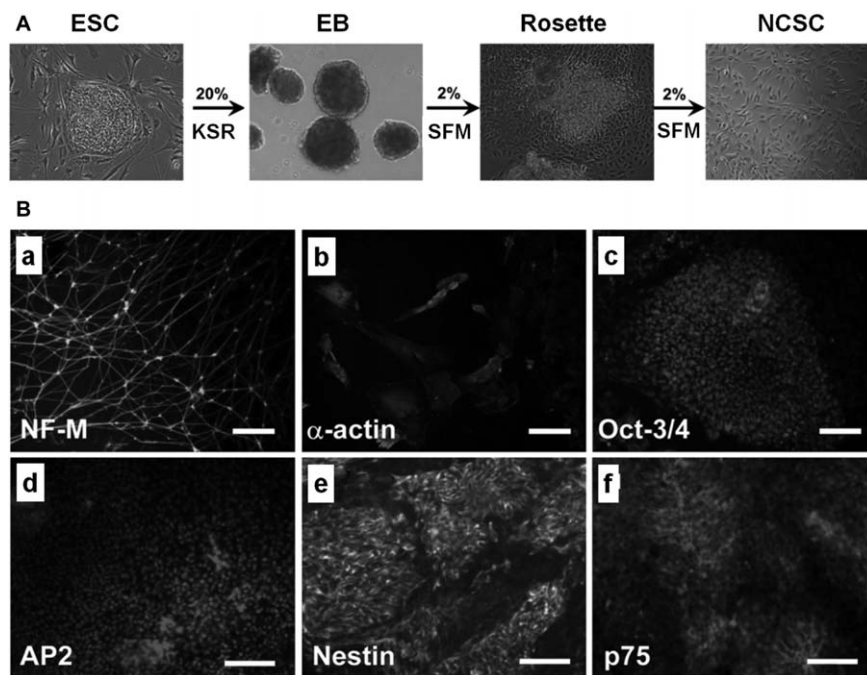


Figure 5.4 NCSCs (neural crest stem cells) obtained from hPS cells. (A) The process for differentiation into NCSCs from hPS cells. (B) Expression of NF-M (Neurofilament M subunit) (a). Immunohistochemical staining for smooth muscle α -actin (b) and Oct-3/4 (c). The majority of cells expressed the neural crest markers AP2 (d), Nestin (e) and p75 (f). Scale bar (a) = 200 μ m. Scale bar (b-f) = 100 μ m.⁵⁰ Adapted from ref. 50 with permission from Elsevier, Copyright 2011.

desired cells within the neural lineage, which do not use EB generation (Type E and G induction protocols).^{16,22–36}

Kriks and his colleagues examined human TH^+ neurons on Matrigel-coated dishes using a floor plate-based protocol, and the neurons could extensively engraft in animal models *in vivo*.¹⁶ From these findings, they discussed that past failures to prepare human TH^+ neurons efficiently were because of incomplete maturation as well as low specification of human midbrain TH^+ neurons.¹⁶

In this Type E induction protocol (Figure 5.3(d)), hPS cells were cultivated in media including two SMAD inhibitors (SB431542 and LDN193189) for 24 h.¹⁶ Then, the cells were cultivated in media including two SMAD inhibitors, FGF-8 and SHH (SHH agonist) from day 1 to day 3. The cells were cultivated in media including CHIR99021 (a GSK3B inhibitor known to strongly activate Wnt signaling), FGF-8, SHH, SB431542, and LDN193189 from day 3 to day 5. Then, the cells were cultivated in media including CHIR99021, FGF-8, SHH, and LDN193189 from day 5 to day 7 and subsequently in media including CHIR99021 and LDN193189 from day 7 to day 11.

The midbrain dopaminergic neuron precursor (midbrain floor plate precursor) was formed by day 11.¹⁶ Subsequently, the cells were cultivated in maintenance media including CHIR99021 to generate midbrain dopaminergic neuron (TH⁺/FOXA2⁺ cell). The midbrain dopaminergic neurons, which could be used for engraftment were generated by day 25 and cultivated for several months *in vitro*. The percentage of dopaminergic neurons was much higher using the floor plate-derived culture protocol (75%) compared to typical rosette-based methods (20–35%).¹⁶ The dopaminergic neurons prepared using both methods indicated NURR1 expression, although only floor plate-derived dopaminergic neurons showed further expression of roof plate marker (LMX1A) and floor plate marker (FOXA2).¹⁶

Although rosette-derived dopaminergic neurons show only little rescuing symptom of rat and mouse model of Parkinson's disease (rat and mouse having lesions with 6-hydroxydopamine), floor plate-derived dopaminergic neurons (TH⁺/FOXA2⁺ cells), which induced from hES cells and grafted into rat or mouse models of Parkinson's disease, indicated almost perfect restoration of amphetamine-induced rotation behaviors and extensive survival.¹⁶ The findings were elucidated by the higher survival period of floor plate-derived dopaminergic neuron (4–5 months) than rosette-derived dopaminergic neuron (1.5 months). Furthermore, a monkey of Parkinson's disease model was transplanted with the floor plate-derived dopaminergic neurons as a scalable model. Significant dopaminergic neuron function and survival and a lack of neural overgrowth were reported in the Parkinson's disease model using a monkey.¹⁶

The dopaminergic neurons, which were derived from the floor plate protocol using hPS cells give hope for the stem cell-based therapies for Parkinson's disease. Gonzalez and his colleagues further examined the step-wise induction protocol of hPS cells into dopaminergic neurons (Type E induction protocol, Figure 5.3(e)).³⁷ In their method, hPS cells were cultivated on Matrigel-coated plates in media including DMH-1 and SB218078 for 7 days. The neuralized hPS cells were cultivated in NS cell media including EGF, FGF-2, and StemPro neural supplement to derive neural stem (NS) cells. The hPS cell-derived NS cells exhibited 96% for Musashi-1, 98% for Nestin, and 95% positive for Pax6 from immunohistochemical staining of the marker proteins after four passages.³⁷ Moreover, hPS cell-derived NS cells were cultivated on Matrigel-coated plates in the media including FGF-8 and purmorphamine for 7 days. Then, hPS cell-derived NS cells were cultivated on Matrigel-coated plates in the media including guggulsterone for 14 days. The differentiated cells showed the expression of high percentage (>95%) of dopaminergic neuron marker of TH on 14 days after treatment with guggulsterone.³⁷

This method can generate extensively high purity of dopaminergic neuron derived from hPS cells, although the total induction time is relative longer (about 2 months) than Kriks' method (less than 1 month).¹⁶ Gonzalez's protocol³⁷ used Matrigel-coated plates for hPS cell induction substrates, while Kriks' protocol¹⁶ did not describe what kind of cultivation plates were used to prepare midbrain dopaminergic neurons.

Furthermore, Lippmann and his colleagues investigated the induction of hPS cells into neuroepithelium using widespread rosette formation (99% N -cadherin⁺/Pax6⁺ cells) with no manual selection step in 6 days using the Type E induction protocol where hPS cells were cultivated on Matrigel-coated plates or recombinant human vitronectin (VN) peptide-coated plates in Essential 6 media (DMEM/F12 media including insulin, transferrin, ascorbic acid, selenium, and sodium bicarbonate).³⁸ The neuroepithelium obtained in this work extensively induced into GFAP⁺ cells (astrocytes) and HB9⁺ cells (motor neurons) (Figure 5.3(f)), which were cultivated on Matrigel-coated plates.³⁸ This completely scalable and defined protocol would be useful for scale-up cultivation for the preparation of therapeutic neural cells in stem cell therapies.

It should be important to study the effect of cell cultivation plates and several materials on the induction of hPS cells into midbrain dopaminergic neuron. Then, it should be important to evaluate the appropriate stiffness of cell culture materials and immobilized nanosegments (*i.e.*, glycosaminoglycan, oligopeptides, and proteins) for cell cultivation surface to facilitate hPS cell induction into midbrain dopaminergic neuron.

5.2.4 Effect of Cell Cultivation Materials on hPS Cell Induction into Neural Lineages

The material–cell interaction is an important factor for controlling the induction of hPS cells. Some researchers examined the effect of cell cultivation materials on hPS cell induction into neural lineages (Table 5.2).^{16,19,22–36,39–60} The grafting or coating biomaterials used in the investigation described in Table 5.2 are depicted in Figure 5.5. PLO/LN, LN, and Matrigel as well as Geltrex were generally used where Matrigel includes components such as enactin, LN, heparan sulfate proteoglycans, collagen IV, and some growth factors, which are isolated from sarcomas in Engelbreth–Holm–Swarm mouse. Matrigel is a xeno-including biomaterial, and not a chemically defined biomaterial. Gelatin (GEL), VN, collagen (COL), and ECM-derived oligopeptides have not been frequently utilized. It would be valuable to study cell cultivation biomaterials immobilized with oligopeptides, which correspond to cell binding sites of desired ECM segments for hPS cell induction into neural lineage.

In this chapter, we examine the effect of several sophisticated materials, which have been reported by some researchers for the induction of hPS cells into neural lineage.^{40,45,49,50,82–84}

SHH works an important role in the development of midbrain dopaminergic neurons and forebrain GABA-inhibitory neurons. Hyaluronic acid (HyA) conjugated with multivalent SHH proteins (HyA-SHH) was developed by Vazin and his colleagues (Figure 5.6),⁴⁹ and the effect of SHH valency on the ventral specification of hES cells into dopaminergic neuron and GABA inhibitory neuron was studied using Type B and D induction protocols.⁴⁹ EB was cultured on Matrigel-coated dishes in medium including SHH-HyA to generate neural rosette cells. Then, the cells were selected and cultured on LN/PLO-coated plates in medium including SHH-HyA to form dopaminergic

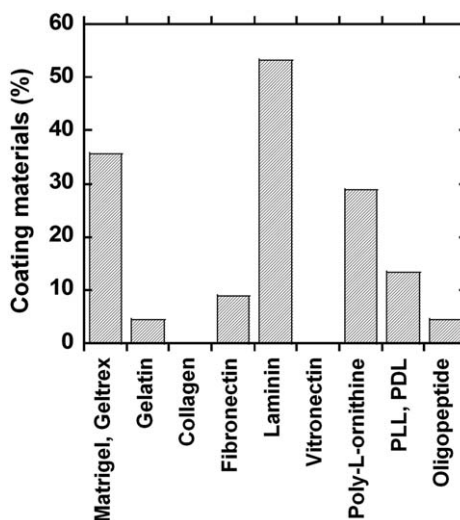


Figure 5.5 The grafting and coating biomaterials used for hPS cell differentiation into neural lineages in 39 studies from 2001 to 2015. PDL, poly-D-lysine; PLL, poly-L-lysine.⁵
Adapted from ref. 5 with permission from Elsevier, Copyright 2016.

neuron (Figure 5.6). GABAergic cells were generated by inducing hES cells in a similar protocol, but FGF-8 was not used during neural induction and GDNF was not included during the maturation into neuron.⁴⁹

HyA immobilized with multivalent SHH with the appropriate valency (sixteen SHH on a SHH-HyA biomaterial) promoted the generation of midbrain dopaminergic neuron from 29–31% to 59–61% in comparison to monomeric SHH.⁴⁹ Furthermore, only 50% of hES cells induced into GABAergic neuron when using traditional monomeric SHH, while 85% of hES cells induced into GABAergic neuron when HyA immobilized with multivalent SHH was included into the cultivation medium.⁴⁹ The findings indicate that HyA immobilized with appropriate multivalent SHH facilitates the induction of hES cells into neuronal lineage, which would be important for the clinical therapies for Parkinson's disease and epilepsy.

For neuroregeneration using iPS cell-derived neural cells, Kuo and his colleagues developed polyacrylamide (PAA)-chitosan (CS) inverted colloidal crystal (ICC) scaffold grafted with PLGA [poly(lactide-co-glycolide)] nanoparticle and transactivator of transcription von Hippel-Lindau (TATVHL) peptide.⁸⁵ The hybrid PAA-CS ICC scaffolds were immobilized with PLGA nanoparticles and TATVHL peptide had regular pore sizes and a rough pore surface for a better induction of iPS cells into neural lineage. The iPS cells cultivated in the scaffolds with PLGA nanoparticles at 1.0 mg mL^{-1} and TATVHL peptide at 15 mg mL^{-1} showed excellent elongation of the axonal length to $15 \text{ }\mu\text{m}$.⁸⁵ A combination of TATVHL peptide and PLGA nanoparticles was found to favor the adhesion of iPS cells and to decrease

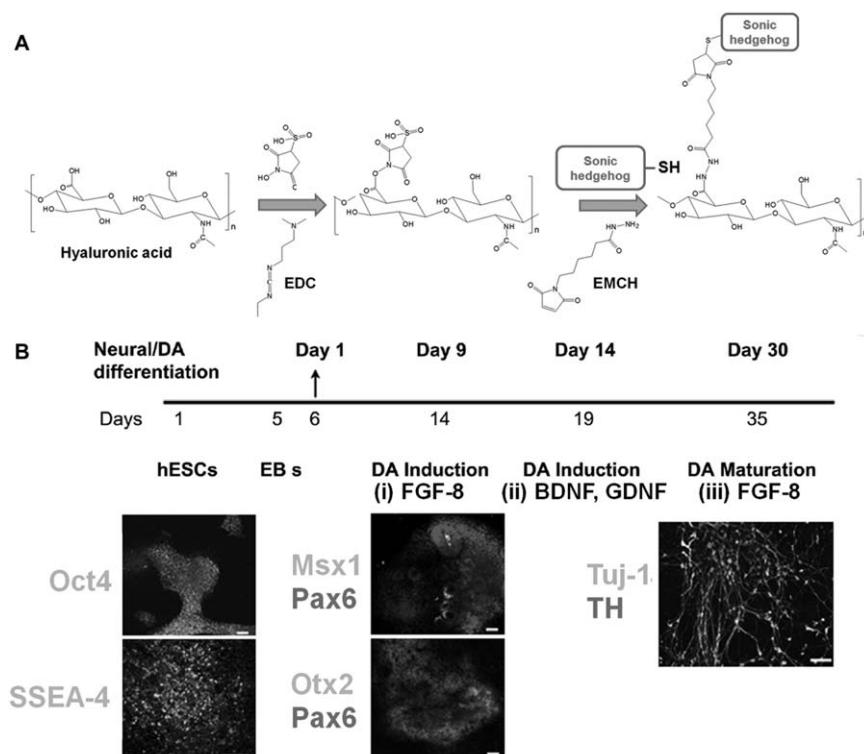


Figure 5.6 Dopaminergic neurons induced from hES cells using multivalent SHH (sonic hedgehog). (A) Chemical scheme for the preparation of HyA-SHH bioconjugate. (B) Schematic of the timeline for the induction of hES cells into midbrain dopaminergic (mDA) neuron with the aid of the midbrain instructive factors, FGF-8 and SHH. Cells were stained for the pluripotent stem cell markers, Oct4 and SSEA4, on the first day, the early neural progenitor marker Pax6 and the midbrain progenitor markers Otx2 and Msx1 on day 14, and dopaminergic (TH) and neuronal (Tuj-1) markers on day 35 to evaluate the DA (dopaminergic) induction of hES cells. Scale bar = 100 μ m.⁸⁷

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pluripotency of iPS cells after cultivation, and led to produce β III tubulin-positive cells in the scaffold. In addition to the induction of differentiation toward neurite-like cells, an enhancement in the content of TATVHL peptide in the scaffold restricted the induction of iPS cells into astrocytes.⁸⁵ ICC scaffolds composed of TATVHL peptide, PLGA nanoparticles, CS and PAA may be an optimal matrix to induce iPS cells into neurons and retard the glial generation for nerve regeneration.

Silva and his colleagues developed fibrin hydrogels grafted with several synthetic peptides engaging integrin $\alpha 6 \beta 1$ (a receptor for LN), a cell receptor enriched in neural stem/progenitor (NPS) cells to facilitate affinity of fibrin

hydrogels towards mouse ES (mES) cell-derived NPS cells.⁸⁶ Six $\alpha 6\beta 1$ integrin ligands, which were A5G81 (AGQWHRVSVRWG), N4 (CGLPYSSVC), P3 (VSWFSRHRYSPPFAVS), HYD1 (KIKMVISWKG), AG10 (NPWHSIYITRFG), and T1 (GTTWSQCSKS) with a C-terminal amide, were evaluated for their ability to promote integrin $\alpha 6\beta 1$ -mediated adhesion of mES cell-derived neural stem and progenitor (NSP) cells and to sustain neuronal differentiation, migration, and viability of mES-NSP cells. Due to their better performance, fibrin hydrogels grafted with peptides A5G81, HYD1, and T1 showed better mES-NSP cell attachment than other fibrin hydrogels and were examined in terms of peptide binding efficiency as well as viscoelastic and structure characteristics.⁸⁶ Immobilization of HYD1 or T1 on fibrin hydrogels successfully promoted cell outgrowth from mES-NSP cell neurospheres (up to 2.4-fold enhancement), which showed a biphasic response to peptide concentration. Inhibition experiments indicated the involvement of $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins in mediating radial outgrowth on fibrin hydrogels grafted with HYD1 and T1 peptides. The immobilization of HYD1 and T1 peptide on fibrin hydrogels also facilitated neurite extension of mES-NSP cells in the hydrogels, with no affecting neuronal differentiation and/or cell proliferation.⁸⁶ Fibrin hydrogels grafted with HYD1 were found to provide a permissive environment for axonal regeneration, which lead up to a 2.0-fold enhancement in neurite extension from rat dorsal root ganglia explants in comparison to unmodified fibrin hydrogels, and to extensive improved BBB (Basso, Beattie, Bresnahan) locomotor function after spinal cord injury of rats (complete transection), along with a trend toward a higher area positive for GAP 43 (growth associated protein 43, a marker for axonal growth cone formation).⁸⁶ Their findings describe that conjugation of $\alpha 3\beta 1$ integrin-binding peptide is powerful to enhance the biofunctionality of hydrogels used in 3D scaffolds for ES-NSP cell cultivation and potentially, in matrix-assisted ES-NSP cell implantation.

A peptide-modified, minimally invasive, injectable hydrogel composed of hyaluronan and methylcellulose was prepared by Führmann and his colleagues,⁶¹ which enhance the survival and differentiation of hiPS cell-derived oligodendrocyte progenitor cells, because transplantation of oligodendrocyte progenitor cells has the potential to regenerate or preserve neural functions after central nervous system injury. hiPS cell-derived oligodendrocyte progenitor cells were implanted sub-acutely after a moderate clip compression rat spinal cord injury.⁶¹ The hydrogel, modified with the RGD peptide and PDGF (platelet-derived growth factor), facilitated early survival and integration of grafted cells. The teratoma generation by transplantation of hiPS cell-derived oligodendrocyte progenitor cells was reduced when the cells were transplanted in the hydrogel, where most cells differentiated to a glial phenotype.⁶¹ The hydrogels promoted cell survival and integration, and reduced teratoma formation by facilitating the cell differentiation.

The conducting polymeric polypyrrole was used as cell cultivation materials for hPS cell induction into neural cells by Zhang and his colleagues.⁴⁵ hES cells were cultivated on polypyrrole surface grafted with oligopeptides

derived from LN (DRNIAEIIKDIC [p20], DCDPGYIGSR [p31], and a mixture of p20 and p31) using the Type F and B protocols. hES cell cultivation on polypyrrole grafted with either p20 or p31 oligopeptides facilitated induction into neuroectoderm after a week. After 2 weeks of culture, only 10% of cells showed expression of β III tubulin after induction on LN-coated plates or on polypyrrole surface grafted with p31, while a high percentage of hES cells, which were cultivated on polypyrrole grafted with p20, induced into neuronal differentiation (25% β III tubulin⁺ cells) (Figure 5.7).⁴⁵ On the other hand, hES cells cultivated on polypyrrole surface grafted with p31 exhibited better cell adhesion and spreading (Figure 5.7).⁴⁵ This findings gives some ideas as to how incorporated bioactive peptides and conductive polymers can contribute to the induction of hES cells into neurons.

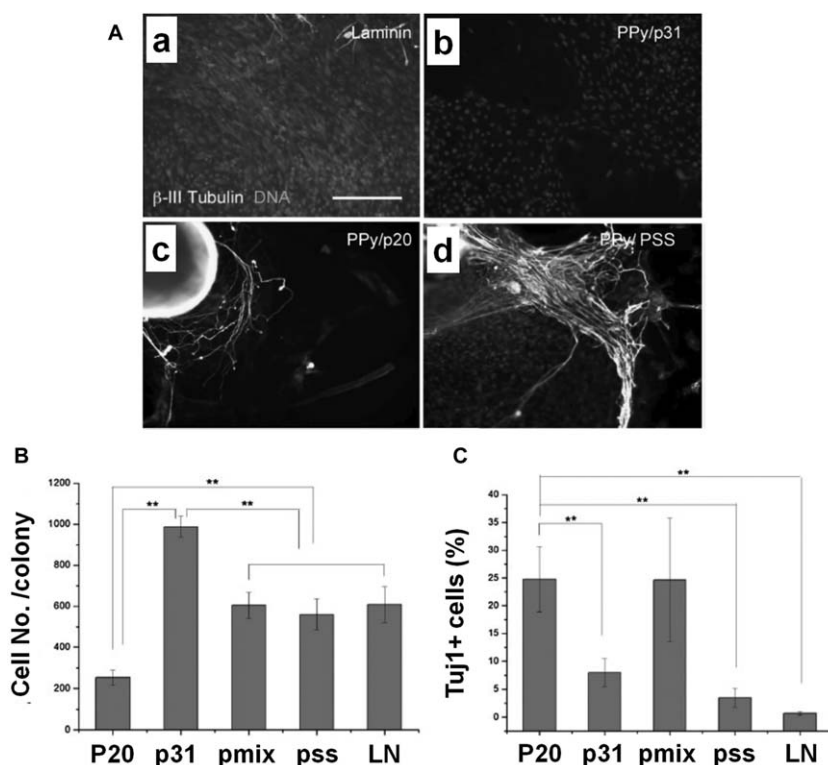


Figure 5.7 hES cell-derived neuronal cells on LN peptide-doped polypyrrole material surface after 2 weeks in cultivation. (A) Cells on various polypyrrole (PPy) surfaces including LN (a), p31 (b), p20 (c), and polystyrene sulfonate (pss) (d) were stained with antibodies against β -III tubulin (bright) and counterstained with Hoechst (nucleus; gray). (B, C) Average cell number per colony (B) and percentage of Tuj1⁺ cells (C) in hES cell-derived neuronal cells on various polypyrrole (PPy) material surface including polystyrene sulfonate (pss), p20 + p31 (pmix), p31, p20, or LN.⁴⁵ Adapted from ref. 45 with permission from John Wiley and Sons, Copyright © 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

The induction of hPS cells into a desired cell lineage is controlled by specific topographical cues.^{4,87} On the other hand, the function of topography has not yet been extensively examined. Then, the effect of topographical designs on the induction of NPS cells into dopaminergic neurons was investigated by Tan and his colleagues.⁸² They manufactured a multi-architecture (MARC) chip composed of stiff TCP plates and soft polydimethylsiloxane (PDMS) plates with several topographical shapes: pillars, wells, hierarchical grating, microlens, and moth eye morphologies on several scales. Cells that express TH (dopaminergic-committed neuron) and TUJ1 (β -III tubulin, immature neurons) were found on grating 1 ($2 \times 2 \times 2 \mu\text{m}$ grating), grating 5 ($250 \times 250 \times 250 \text{ nm}$), and grating 6 ($2 \times 2 \times 2 \mu\text{m}$ grating and $250 \times 250 \times 250 \text{ nm}$ line) made of PDMS (Figure 5.8).⁸² The cell cultivation biomaterials in gratings 5 and 1, on which NPS cell induced into large percentages of TH- and TUJ1-expressing cells, had a grating aspect ratio of 1 (height:spacing:grating width = 1:1:1). The induction of NPS cells into dopaminergic neurons was decreased by reducing the aspect ratio (gratings 2–4) (Figure 5.8).⁸² Then, the aspect ratio of the cell cultivation biomaterials is a key point for controlling the specific differentiation of NPS cells.

The tendencies of NPS cells to induce into dopaminergic neurons on the topographical materials composed of stiff polystyrene (elastic modulus 2150 MPa) were similar to the tendency on the topographical materials composed of soft PDMS (3 MPa). This finding indicates that the topographical properties generated by cell cultivation biomaterials play a more important task than the elasticity of cell cultivation biomaterials, although Engler and his colleagues reported the significant effect of cell cultivation hydrogel stiffness on stem cell fate.^{88–90}

Electrospinning fibers show well-defined micro/nanofiber morphologies having regulated alignments, radii, and surface characteristics. The topographic cues given by electrospinning fibers, such as radius and alignment, as well as the fiber material provide the influence into the differentiation fate of hPS cells in differentiation medium.^{44,46,83,84}

Mahairaki and his colleagues investigated hES cell induction into neurons on electrospinning PCL (polycaprolactone) fibers with varied orientations (*i.e.*, random or aligned fibers) and several diameters ($1 \mu\text{m}$ to 250 nm), which were coated with PLO and LN to promote the neural cell adhesion.⁸³ Neural precursors obtained from hES cells, which were cultivated on conventional flat TCP plates or on random micro/nanofibers, exhibited nonpolarized neurite networks, while the neural precursors on aligned micro/nanofibers showed polarized cell morphologies along the axes of the aligned micro/nanofibers.⁸³ The neural precursors on aligned micro/nanofibers displayed extensive neuronal induction in comparison to the neural precursors on random micro/nanofibers or flat TCP plates. In total, 60% and 85% of hES cell-derived neuronal precursors showed the expression of β III tubulin (early neuronal marker) when the hES cells were cultured on aligned microfibers or nanofibers, respectively, while only approximately

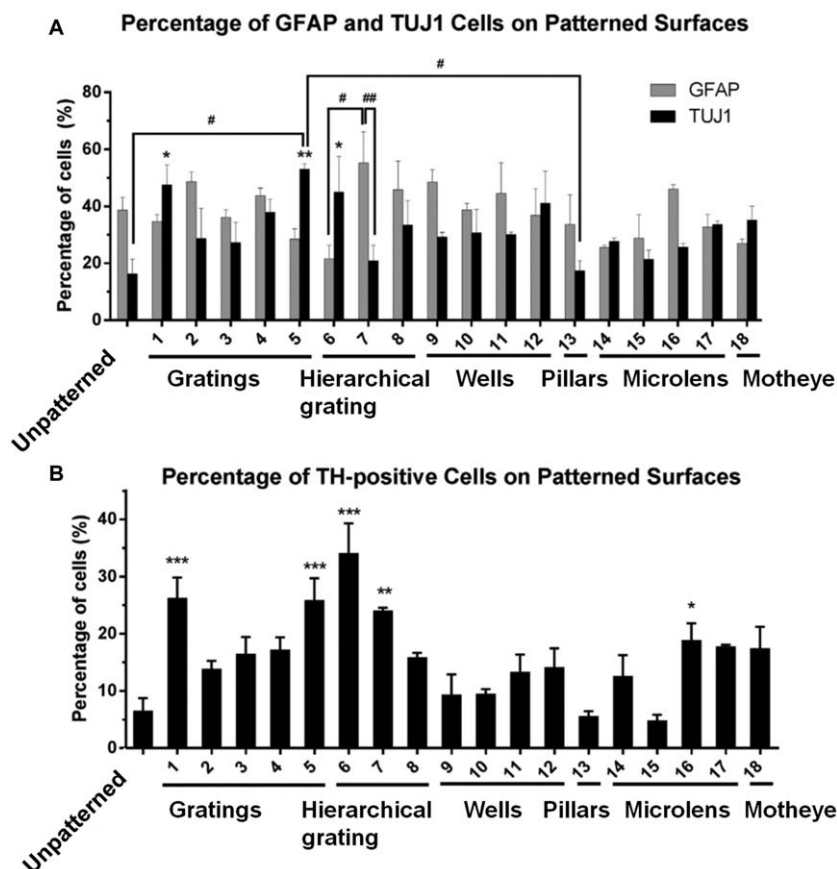


Figure 5.8 Screening with an 18 pattern MARC (multi-architecture) chip for neural progenitor cell induction. (A) Induction of neural progenitor cells into TUJ1 (β -III-tubulin; neurons)- and GFAP (glial fibrillary acidic protein; astrocytes)-positive cells on MARC chips. There was a higher percentage of TUJ1-positive cells prepared on $2 \times 2 \times 2 \mu\text{m}$ gratings (1), $250 \times 250 \times 250 \text{ nm}$ gratings (5), and $2 \times 2 \times 2 \mu\text{m}$ gratings with $250 \times 250 \times 250 \text{ nm}$ lines (6) than on an unpatterned material surface. (B) Induction of neural progenitor cells into TH (tyrosine hydroxylase; dopaminergic)-positive cells on MARC chips. There was a greater percentage of TH-positive cells prepared on $2 \times 2 \times 2 \mu\text{m}$ gratings (1), $250 \times 250 \times 250 \text{ nm}$ gratings (5), and $2 \times 2 \times 2 \mu\text{m}$ gratings with $250 \times 250 \times 250 \text{ nm}$ lines (6) than on an unpatterned material surface.⁸² Adapted from ref. 82 with permission from Elsevier, Copyright 2015.

30% of neuronal precursors showed the expression of β III tubulin when the hES cells were cultivated on both random microfibers and nanofibers.⁸³ The neural precursors obtained from hPS cells cultivated on aligned nanofibers that are made from typical polymers or coated with any biomaterial, facilitate neuronal induction in general.^{44,46,83,84}

Ren and his colleagues also investigated the effect of the fiber topography (different alignment and diameter) of electrospinning polysulfone micro/nanofibers on the induction of hES cells into neural crest stem cells towards a Schwann cell lineage.⁸⁴ The aligned fibers provided great cell alignment. hES cells cultivated on aligned fibers with an average diameter of 1.6 μm or 600 nm strongly induced into neural crest stem cells towards a Schwann cell lineage in comparison to hES cells cultivated on random fibers (diameter: 1.6 μm , 600 nm, or 160 nm), to hES cells cultivated on aligned fibers with a diameter of 160 nm or to hES cells on flat 2D TCP plates.⁸⁴ Therefore, the induction fate of hPS cells depends on fiber topography (*i.e.*, random or aligned fibers) as well as on fiber diameter. Moreover, fibers >500 nm in diameter facilitated the induction of hES cells into Schwann cells, whereas fibers <500 nm in diameter promoted the induction of hES cells into NS cells and neural precursors.

Some biodegradable synthetic polymers, such as PLGA and PLLA [poly(L-lactic acid)] have been studied as scaffold for neural cells.^{40,44,50} Levenberg and his colleagues investigated the neuronal induction of hES cells on PLLA scaffolds with a pore size of 200–500 μm and on Matrigel-coated PLGA to create neural-like tissues *in vivo* using the Type A induction protocol.⁴⁰ A high ratio of the cells (21% of the tissue) inoculated from EB in the scaffold showed the expression of $\beta\text{III-tubulin}$ in this research. Vascularization was found throughout the scaffold, which was analyzed from an endothelial cell marker of CD31 expression. The presence of retinoic acid in the cultivation medium inhibited endothelial induction as well as endothelial vessel development.⁴⁰ This study might indicate the potential of hPS cell-based tissue engineering for the therapy of damaged neural tissue.

Wang and his colleagues obtained neural crest stem cells from hiPS cells and hES cells using the Type D induction protocol. They made a nerve conduit where neural crest stem cells were inoculated on aligned poly(L-lactide-co-caprolactone) nanofibers.⁵⁰ The nerve conduit connected transected sciatic nerves in rats (Figure 5.9).⁵⁰ Sciatic nerve regeneration was facilitated in the rats transplanted with nerve conduits entrapped with neural crest stem cells after 30 days. Especially, axonal myelination was promoted by the implantation of neural crest stem cell. Moreover, Schwann cell induced from neural crest stem cell was found to be merged into the myelin sheath around axon.⁵⁰

These data suggest that neural crest stem cells induced from hiPS cells and hES cells in combination with biodegradable polymer scaffold might be valuable in tissue engineering.

5.3 Induction of hPS Cells into Cardiomyocytes

The effective preparation methods of human cardiomyocyte from hES cells and hiPS cells are reported currently, which would contribute predictive toxicology and drug screens and to cardiac therapies.^{91,92} In this session, we evaluate the currently developed protocol for effectively inducing hPS cells into cardiomyocyte^{17,18,91,93,94} and the effect of cell cultivation materials on the induction of hPS cells into cardiomyocyte.^{18,95–97}

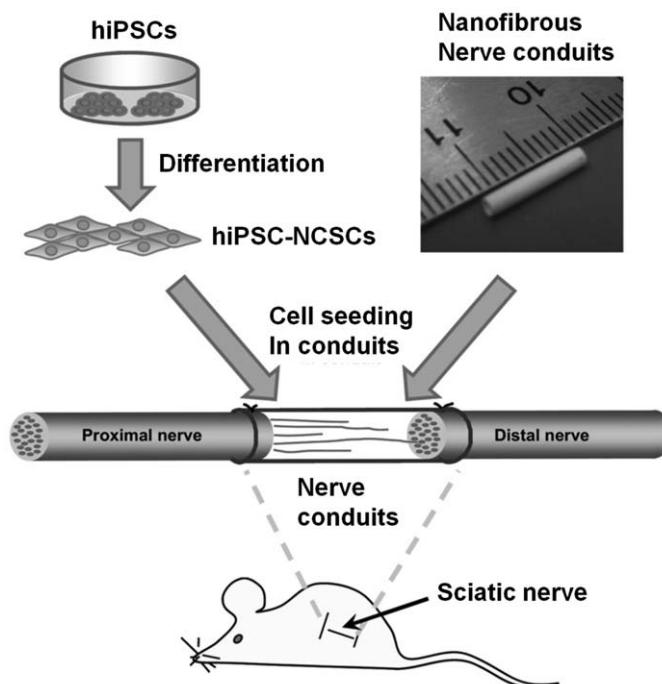


Figure 5.9 Peripheral nerve regeneration using tissue-engineered nanofibrous nerve conduit. Schematic picture of the tissue engineering approach of a combination of nanofibrous nerve conduit and NCSCs (neural crest stem cells). The NCSCs mixed with Matrigel were inserted into the nerve conduit and cultured for 24 h *in vitro*.⁵⁰ Adapted from ref. 50 with permission from Elsevier, Copyright 2011.

5.3.1 Efficient Protocols for Inducing hPS Cells into Cardiomyocyte

The induction of hPS cells into cardiomyocyte is generally considered using following two processes: (1) mesoderm differentiation through the FGF, BMP, Wnt, and Activin-Nodal pathways; and (2) subsequent cardiac specification through the restriction of the Wnt, BMP, and TGF- β pathways.¹⁸ Several investigations of cardiomyocyte induction are shown in Table 5.3,^{17,18,91,93–129} and some of the induction methods are described in Figure 5.10. Moreover, some investigators proposed cardiomyocyte sorting methods, which produced an excellent purity (>95%) of cardiomyocyte. SIRPA (CD172a)-positive sorting by FACS or MACS would produce an excellent purity of cardiomyocyte (for example, >97% of cTnT⁺ cells), as investigated by Zhang and his colleagues⁹¹ or Dubois and his colleagues⁹³ using the Type D induction protocol (Figure 5.10(d)).

Conventional cardiomyocytes generated from hPS cells have less cardiac function in comparison to adult human myocardium having 40–50 cm s⁻¹

Table 5.3 Differentiation of hPS cells into cardiomyocytes cultured on biomaterials.⁵ Adapted from ref. 5 with permission from Elsevier, Copyright 2016.

hPSCs	Cell culture materials	Methods	Cell type (%)	Ref. (year)
hESC	Agarose hydrogels	Type AB	Cardiomyocytes (cTnT cells, 65%)	98 (2013)
hESCs, hiPSCs	Suspension	Type AB	Cardiomyocytes	99 (2015)
hESCs (SNUhES31)	Gold-coated PCL	Type AB	Cardiomyocytes	100 (2015)
hESCs (BG02, H9)	Suspension	Type AB	Cardiomyocytes (cTnT ⁺ cells, 95.7%)	101 (2015)
hiPSCs	Pluronic F127-coated plate	Type AB	Cardiomyocytes	125 (2017)
hESCs (HES2, HES3), hiPSCs (MSC-iPS1)	Ultra-low attachment plate	Type AB	Cardiomyocytes	127 (2017)
hESCs (HES2, H7, and H9), hiPSCs	Ultra-low binding plate	Type AB	Cardiomyocytes	129 (2014)
hESCs (HES2)	Gelatin sponge scaffolds	Type A	Cardiomyocytes	94 (2014)
hESCs, hiPSCs	Polyacrylamide gel with gelatin line pattern	Type A	Cardiomyocytes	99 (2015)
hESCs(H9.2)	Gelatin-coated dishes and PEGylated fibrinogen hydrogels	Type B	Cardiomyocytes	102 (2009)
hESC (H9)	Matrigel-coated polyurethane dishes	Type B	Cardiomyocytes	103 (2010)
hEDS (H9)	Elastin-like hydrogels	Type B	Cardiomyocytes	104 (2012)
hESCs (H1, H9)	Gelatin, fibronectin, or collagen IV coated dishes	Type B	Cardiomyocytes (cTnT ⁺ cells, 14%)	105 (2012)
hESCs (H9, H13, H14), hiPSCs (6-9-9, 19-9-11)	Gelatin-coated dishes	Type B	Cardiomyocytes	106 (2012)
hESCs (H1, H9, UCLA1-6)	Oxygen-treated PDMS dishes	Type B	Cardiomyocytes	107 (2013)
hESCs	Collagen hydrogels with human fibroblasts or hBMSCs	Type B	Cardiomyocytes	130 (2017)
hESCs (HUES7)	Polymer microarray	Type C	Cardiomyocytes	108 (2015)
hESCs (HES2)	TCPS	Type D	Cardiomyocytes (cTnT ⁺ cells, 98%)	93 (2011)

hESCs (HES-2)	Fibrin patch or fibrin-coated dishes	Type D	Cardiomyocytes (SIRPA ⁺ cells, 48–90%)	91 (2013)
hESCs (H7)	VN-derived oligopeptide-grafted polyacrylate dishes	Type E	Cardiomyocytes (Nkx2.5 ⁺ cells 60%, α -actinin ⁺ cells, 60%)	109 (2010)
hESCs (H9)	PLL-coated alginate hydrogels	Type E	Cardiomyocytes (cardiac cells)	110 (2010)
hESCs (HES-3, H1)	LN-coated microcarriers (Toso-10 MC, DE-53, Cytodex 1, Cytodex 3 FACT)	Type E	Cardiomyocytes (MHC ⁺ cells, 17%)	96 (2010)
hESCs (HES2)	Aligned Matrigel-coated polyethylene plates having groove	Type E	Cardiomyocytes	111 (2013)
hESCs, hiPSCs (6-9-9 ishcat-1, 19-9-11)	Matrigel or Synthemax dishes	Type E	Cardiomyocytes	17 (2013)
hESCs (H9, H13, H14), hiPSCs (6-9-9, 19-9-11)	Matrigel-coated dishes	Type E	Cardiomyocytes	106 (2012)
hESCs (H9)	Pullulan/dextran/fucoidan/gelatin scaffolds	Type E	Cardiomyocytes	112 (2014)
hESCs (H9)	Matrigel or fibronectin coated polyacrylamide hydrogels	Type E	Cardiomyocytes (cTnT ⁺ cells, 52%)	113 (2014)
hESCs (HES-3), hiPSCs (IMR-90)	Cytodex 1 microcarriers coated with Matrigel	Type E	Cardiomyocytes	114 (2014)
hESCs (H7, H9), hiPSCs (59FSDNC3),	Synthemax dishes	Type E	Cardiomyocytes (cTnT ⁺ cells, 95%)	18 (2014)
hESCs (WA09), hiPSCs (IMR90)	Matrigel-coated dishes	Type E	Cardiomyocytes	115 (2015)
hESCs (H7)	Matrigel-coated polyacrylamide hydrogels	Type E	Cardiomyocytes	97 (2015)
hESCs (HUES7)	Polymer microarray	Type E	Cardiomyocytes	108 (2015)
hiPSCs	Matrigel-coated dishes	Type E	Cardiomyocytes	116 (2015)
hiPSCs (CC2)	Matrigel-coated dishes and electrospun PEG- <i>b</i> -PCL-co-PCL coated with VN	Type E	Cardiomyocytes	117 (2015)
hiPSCs (WTC-11, GCaMP)	Matrigel-coated plate, decellularized ECM derived from bovine heart	Type E	Cardiomyocytes	136 (2016)
hiPSCs	Matrigel-coated plate, rat decellularized ECM	Type E	Cardiac patch	135 (2016)

Table 5.3 (Continued)

hPSCs	Cell culture materials	Methods	Cell type (%)	Ref. (year)
miPSCs	Matrigel-coated plate	Type E	Cardiomyocytes	128 (2016)
hESCs (SNUhES3)	Recombinant VN-coated plate	Type E	Cardiomyocytes	126 (2017)
hiPSCs	Matrigel-coated plate, fibrin hydrogels	Type E	Cardiomyocytes	124 (2017)
miPSCs (iPS-MEF-NG-178B-5)	Polyacrylamide (9, 20, or 180 kPa), collagen type I, gelatin, or fibronectin-coated plates	Type E	Cardiomyocytes	137 (2018)
hESCs (H9)	Fibronectin-coated plate	Type E	Cardiomyocytes	120 (2018)
hiPSCs (WT11, IMR90)	Matrigel-coated plate and fibrin hydrogels with human dermal fibroblasts	Type E	Cardiac tissue	132 (2018)
hiPSCs (WTC), hESCs (H9)	Micropatterned Matrigel-coated plate on micropatterned PEG-grafted surface	Type E	Cardiac organoids	131 (2018)
hESCs (H7)	Nanofiber hydrogel coated with hyaluronic acid	Type F	Cardiomyocytes	118 (2013)
hiPSCs	Geltrex-coated plate, in neonatal rat hearts	Type E, Type F	Cardiomyocytes	(2017)
hESCs (H9)	Matrigel-coated dishes	Type H	Cardiomyocytes (cTnT ⁺ cells, 98%)	95 (2014)
hESCs	Suspension	Type H and Type AB	Cardiomyocytes	119 (2011)
hESCs	Matrigel-coated dishes	Type H and Type E	Cardiomyocytes	119 (2011)

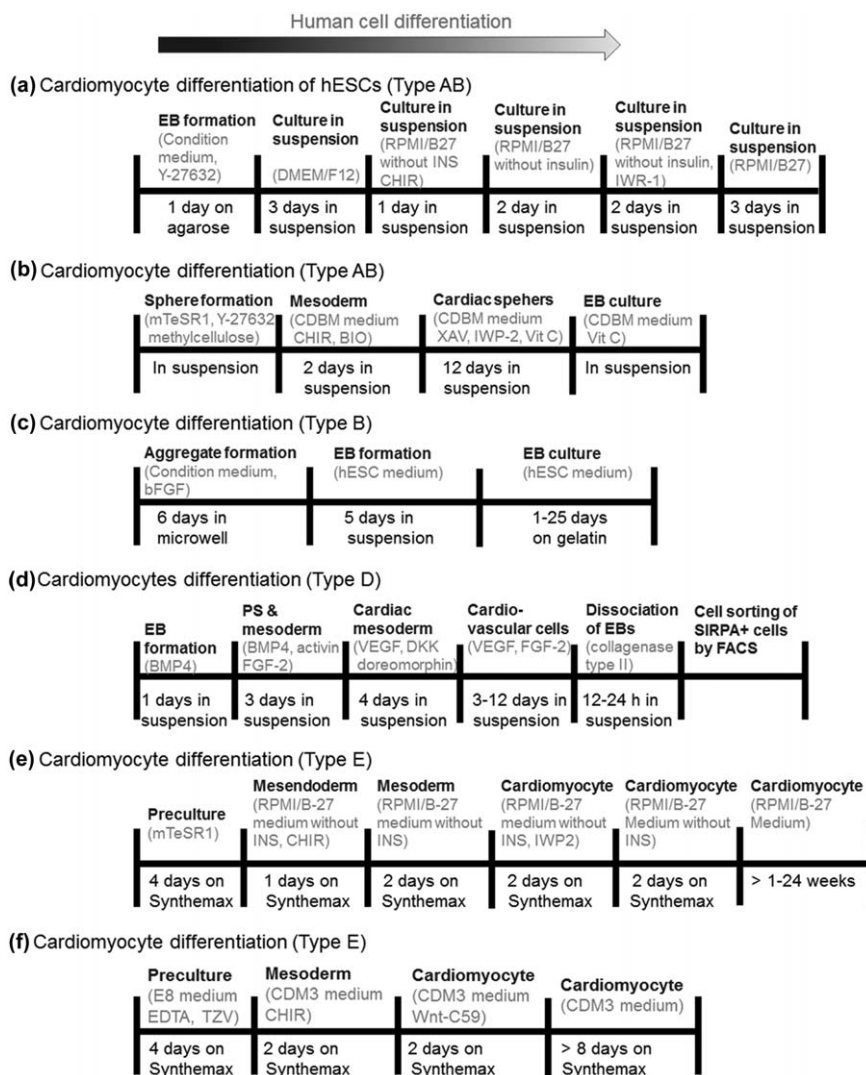


Figure 5.10 Timelines of the protocols for differentiation of hPS cells into cardiomyocytes: (a) the Type AB induction protocol studied by Dahlmann and his colleagues,^{5,98} (b) the Type AB induction protocol studied by Jiang and his colleagues,^{5,101} (c) the Type B induction protocol studied by Mohr and his colleagues,^{5,103} (d) the Type D induction protocol studied by Dubois and his colleagues,^{5,93} (e) the Type E induction protocol studied by Lian and his colleagues,^{5,17} and (f) the Type E induction protocol studied by Burrridge and his colleagues.^{5,18} Adapted from ref. 5 with permission from Elsevier, Copyright 2016.

CV (conduction velocity) and $20\text{--}45\text{ nM mm}^{-2}$ contractile stress. Then, Zhang and his colleagues developed cardiomyocytes using the Type D induction protocol and purified the cardiomyocyte fraction by MACS using the

cardiac marker of SIRPA,⁹¹ which was an originally developed protocol reported by Dubois and his colleagues.⁹³ Zhang and his colleagues made a 3D fibrin-based cardiac patch (Figure 5.11). hES cell-derived cardiomyocytes in this patch showed longer sarcomeres (2.1 μm) and a much higher CV (25.1 cm s^{-1}) in comparison to hES cell-derived cardiomyocytes obtained in 2D culture system. This research suggests the advantages of 3D cardiac patch cultivation in comparison to typical 2D cultivation of hES cell-derived cardiomyocytes.⁹¹ 3D cultivation of hES cells looks like to facilitate the induction of hES cells into cardiomyocytes in comparison to 2D cultivation.

Jiang and his colleagues examined an easy, growth factor-free 3D cultivation protocol to generate 84% cardiac contractile clusters from hiPS cells that were cultivated in suspension to form clusters on ultra-low adhesive plates. hiPS cell spheres were cultivated in suspension in cardiomyocyte differentiation media using the method depicted in Figure 5.10(b). Along with small molecule-based 3D induction, this induction method contributed to produce cardiac spheres from up to 96% of the cardiomyocytes, and 81% of the cardiac spheres supported spontaneous contractility for more than 90 days (Figure 5.12).¹⁰¹ The cardiac spheres expressed pharmacological responses, gene expression, and optimal ultrastructure, which were important for mature cardiomyocyte.

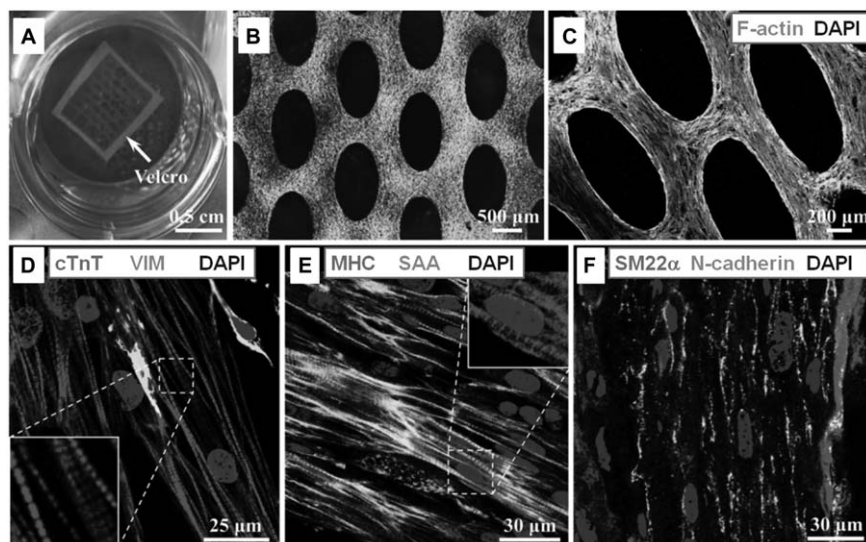


Figure 5.11 Tissue patches of human cardiomyocytes induced from hES cells. (A) Cardiac tissue patch with a Velcro frame after cultivation for 2 weeks. (B) Staggered elliptical pores within the tissue patch. (C) Immunohistochemical staining of F-actin on the cardiac patch. (D) Cardiac patch stained for VIM (vimentin) and cTnT (cardiac Troponin T). (E) Cardiac patch stained for SAA (sarcomeric α -actinin) and MHC (myosin heavy chain). (F) Cardiac patch stained for N-cadherin and SM22 α (smooth muscle cells). DAPI counterstaining was used to observe cell nuclei.⁹¹ Adapted from ref. 91 with permission from Elsevier, Copyright 2013.

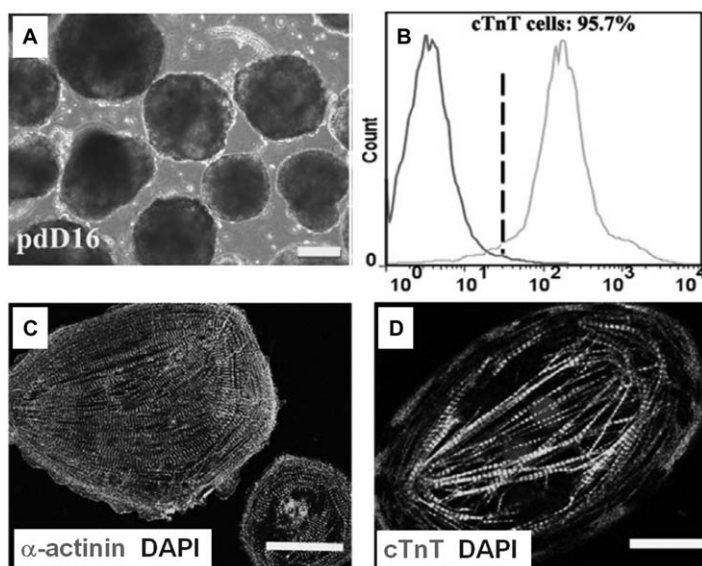


Figure 5.12 Contractible cardiac sphere generated from hPS cells by a small molecule-based 3D cultivation condition. (A) Phase contrast microscopic image of cardiac spheres. Scale bar = 200 μm . (B) Flow cytometric spectra of cells showing cTnT. The left line shows cells stained with the isotype antibody (negative control). (C, D) Preparation of spontaneously beating cardiac sphere showing cTnT and α -actinin. Monolayer adhesive cardiomyocyte prepared from cardiac sphere showed rich sarcomere and multinucleation when dissociated into single cells and cultivated on LN-coated dishes. The cells were stained with antibodies against α -actinin (C) and cTnT (D). DAPI counterstaining was used to observe cell nuclei. Scale bar = 50 μm .¹⁰¹ Adapted from ref. 101 with permission from Elsevier, Copyright 2011.

Multinucleation and polyploidy are specific properties of mammalian cardiomyocyte. Then, cardiac clusters generated in 3D cultivation conditions after 30 days of induction were separated into single cells and cultivated on LN-coated surface for another 48 h. The cells were immunostained with α -actinin and cTnT marker proteins (Figure 5.12).¹⁰¹ 3D-derived cardiomyocyte expressed excellent sarcomere structure, and 30–50% of cardiomyocytes were found to be multinucleated (Figure 5.12).¹⁰¹ These findings suggest that the chemically defined 3D induction method makes the excellent differentiation of hiPS cells into cardiac clusters with extremely enriched cardiomyocyte with no sorting. The cardiomyocytes derived in 3D cultivation system show more mature phenotypes than the cardiomyocytes generated in 2D cultivation system.

Mihic and his colleagues studied the effects of cyclic stretch of cell culture materials on the induction of hES cells into cardiomyocyte.⁹⁴ Mechanical stimulation of hES cells cultivated on stretchable soft materials would be beneficial for induction of hES cells into cardiomyocyte with high purity.

Natural VCMs (ventricular cardiomyocytes) are aligned into extensively organized tissues, which lead to anisotropic electrical conduction for co-ordinated contraction. On the other hand, conventional hPS cell-derived cardiomyocyte clusters are randomly and heterogeneously organized. Therefore, the conventional hPS cell-derived cardiomyocyte clusters can not be representative of natural VCMs. Then, Wang and his colleagues considered and manufactured engineered aligned hES cell-derived cardiomyocyte on biomimetic groove, which had physiologically relevant responses.¹¹¹ The multi-scale wrinkle materials were designed as follows: (1) PE (polyethylene) film was treated with oxygen plasma; (2) the plasma-treated PE film was constrained on opposite sides; and (3) the PE film was elevated temperature at 150 °C to shrink the PE film and to prepare multi-scale aligned wrinkles.¹¹¹ hES cell-derived cardiomyocytes were cultivated on isotropic Matrigel-coated PE film or on PE film with aligned wrinkles, which created isotropic and aligned cardiomyocytes, respectively. Aligned and not isotropic cardiomyocytes showed extensive transverse (T) and longitudinal (L) CVs that were similar to the natural human ventricular anisotropic ratio ($LCV/TCV = 1.8-2.0$).¹¹¹ The total incidence of inducible and spontaneous arrhythmias reduced from 60% for non-aligned cardiomyocyte to 15–25% for aligned cardiomyocyte that gave a physiological baseline for evaluation of arrhythmogenicity. These findings indicated that the anisotropy-induced electrical stability was not because of the mature cellular characteristics of hES cell-derived cardiomyocytes, and might be because of the physical alignment of cardiomyocytes derived from hES cells.¹¹¹ The functional anisotropic hES cell-VCMs prepared by multi-scale topography describes a more precise model for arrhythmogenicity screening of pharmacological factors and drug discovery. This protocol might also contribute to future implantable prototypes with high safety and efficiency against arrhythmias.

Traditional and previous protocols for induction of hPS cells into cardiomyocytes used undefined media (*e.g.*, DMEM/F12 or DMDM media containing FBS [fetal bovine serum]), which hampers clinical use and the examination of the precise molecular mechanism of cardiomyocyte development. Then, Lian and his colleagues induced hPS cells into cardiomyocytes by regulating Wnt/ β -catenin signals under chemically defined conditions using the Protocol E induction protocol (Figure 5.10(e)).¹⁷ They cultivated hPS cells on Synthemax (oligovitronection-grafted acrylic polymer)-coated or Matrigel-coated plates in RPMI/B-27 media containing no insulin during cardiac induction where the insulin in the B-27 supplement is known to inhibit cardiomyocyte differentiation during the first 5 days of induction. The cells were cultured with CHIR99021 (GSK3B inhibitor) for the first day of induction and subsequently treated with IWP2 (Wnt inhibitor) for another 48 h (Figure 5.10(e)).¹⁷ Using this induction method, an extensively pure ratio ($cTnT$ expression = 80–98%) of functional cardiomyocytes derived from hPS cells was generated in two weeks from multiple hPS cell lines with no cell selection or sorting.

Burridge and his colleagues further reported cardiomyocytes derived from hPS cells under chemically defined system using Protocol E.¹⁸ This method is

shown in Figure 5.10(f); the basal RPMI 1640 media including L-ascorbic acid 2-phosphate and rice-derived recombinant human albumin (this is the replacement of bovine serum albumin (BSA) to make a xeno-free component) along with some small molecules, such as Wnt-C59 (Wnt inhibitor) and CHIR99021 (GSK3B inhibitor). Using this method, a contractile cardiomyocyte sheet holding 95% cTnT⁺ cells was generated. The chemically defined method for cardiomyocyte differentiation from hPS cells is an important method for clinical therapy and the study of the molecular mechanism, which is responsible for cardiomyocyte maturation and specification.

hPS cell-derived cardiomyocytes (hPS-CM) are regarded as an attractive cell source for analysis of disease modeling, drug screening and translational medicine. However, hPS-CM are typically in an immature state from consideration of their electrophysiological and contractile structural characteristics. Therefore, Zhang and his colleagues developed cardiac muscle strips by encapsulating hES cell-derived CM (hES-CM) in COL-based biomaterials.¹³⁰ The encapsulation of hES-CM with supplementation of niche cells (human fibroblasts or human bone marrow stem (hBMS) cells) at 3% to the number of hES-CM enhanced the maturation of the hES-CM in 3D tissue matrices. The merit of addition of human mesenchymal stem (hMS) cells and human fibroblasts were found to be comparable for both conditions where the addition of these two different cell types demonstrated similar effects in promoting the cell spreading and compaction, as well as the expression of maturation gene and protein markers.¹³⁰

Mechanical loading, especially cyclic stretching, led to engineered cardiac tissues with high maturity from the evaluation of molecular signature, sarcomere length, and elastic modulus, when comparing to non-stretched or static stretch conditions.¹³⁰ This study indicates that the combination with addition of niche cells and mechanical stretching promotes the maturation of hES-CM in 3D architecture.

Current approaches to generate cardiac organoid are based on either (a) direct cardiac induction from EB or (b) preparation of aligned cardiac tissues from predifferentiated cardiomyocytes from 2D culture of hPS cells. Hoang and his colleagues developed the protocol combining material-based cell patterning with stem cell organoid engineering to generate early cardiac organogenesis *in vitro*.¹³¹ 3D cardiac microchambers were created from 2D hiPS cell colonies; the microchamber approximates an early-development heart with appropriate self-assembly and spatial organization. In this study, micropatterned biomaterials were fabricated using a lithography technique and surface immobilization of polyethylene glycol (PEG) (Figure 5.13).¹³¹ Then, hiPS cell suspensions were seeded into fabricated micropatterns and expanded on the micropatterned surface. Subsequently, the cells were induced following a conventional cardiac differentiation method. The generation of beating cardiac microchamber was detected after 15 days of differentiation. The cardiac microchamber can be evaluated *via* immunocytochemistry with cardiomyocyte markers and the physiological contraction of the beating chambers can be evaluated quantitatively.¹³¹

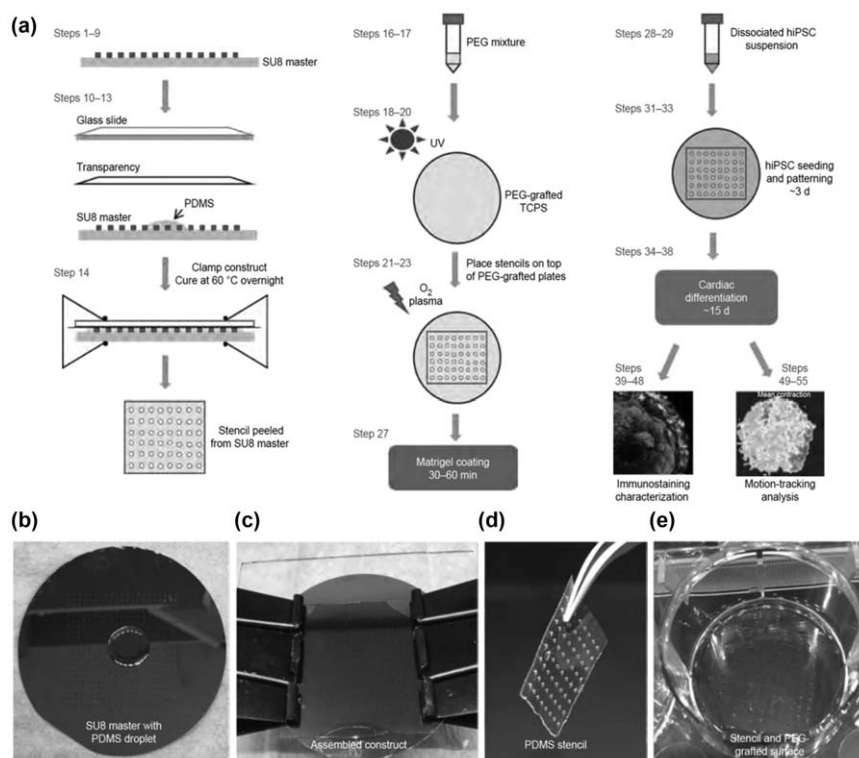


Figure 5.13 Schematic of the procedure and process of PDMS stencil fabrication. (a) Schematic outlining key steps of the process. (b) SU8 master with a small amount of PDMS prepolymer. (c) Completed assembly of the patterned SU8 master-PDMS-transparency-glass slide construct. (d) Thin film of PDMS deleted from the assembly after curing and then located on top of the optically clear PEG-grafted interface (e).¹³¹ Adapted from ref. 131 with permission from Springer Nature, Copyright 2018.

This protocol merges organoid ideas with cell micropatterning method to (a) facilitate the self-organization of 3D tissue structure to mimic early heart and organ formation; (b) provide biophysical cues during cardiac induction of hiPS cells to promote spatial organization of cardiac tissue patterns and cellular polarity; and (c) decrease tissue heterogeneity to generate a reproducible and systematic cardiac organoid model *in vitro*.¹³¹

Cardiac tissues prepared from hiPS cells could contribute to the platforms for patient-specific investigations of disease and physiology. Therefore, Ronaldson-Bouchard and his colleagues prepared cardiac tissues, which were generated from early-stage hiPS cell-derived cardiomyocytes soon after the initiation of spontaneous contractions.¹³² Cardiac tissues were assembled in a modular tissue platform, which could enable individual regulation of the physical signaling and cultivation environment. More precisely, hiPS

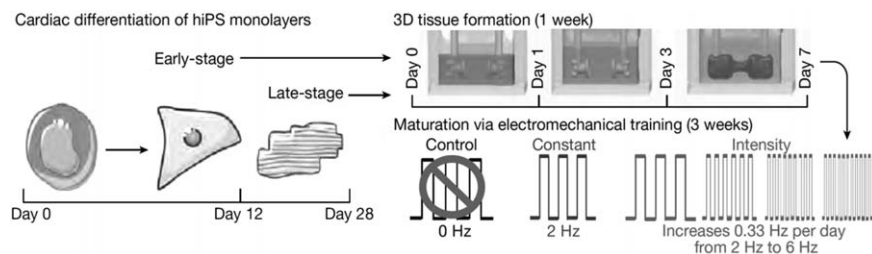


Figure 5.14 Intensity training of cardiac tissue prepared from early-stage hiPS-CMs increases maturation. a, Experimental process: late-stage or early-stage hiPS-CMs and supporting fibroblast were entrapped in fibrin hydrogels to generate tissues stretched between two elastic pillars and made to contract by electrical stimulation. Gradual enhancement in frequency of stimulation to supra-physiological levels (intensity regime) was compared to stimulation at steady frequency (constant regime), unstimulated controls and human adult and fetal heart ventricles.¹³² Adapted from ref. 132 with permission from Springer Nature, Copyright 2018.

cell-derived cardiomyocytes and supporting fibroblasts were entrapped into fibrin hydrogels stretched between two flexible pillars where the mechanical forces were provided to be similar to those in natural myocardium and subjected to electrical stimulation to generate auxotonic contractions.¹³² The size of their resulting tissue was as big as 1.8 mm in diameter and 0.6 cm long. Three conditioning schemes were performed: (a) control (no stimulation); (b) constant (21 days at 2 Hz); and (c) intensity training (14 days at a frequency increasing from 2 Hz to 6 Hz by 0.33 Hz per day, followed by 7 days at 2 Hz) (Figure 5.14).¹³² The early-stage intensity-trained tissues had an excellent electrophysiological characteristics including the shape of the action potential, the resting membrane potential and the CV. Early-stage intensity-trained tissues showed a positive force–frequency relationship, a hallmark of maturation. The $MLC2v^+ : MLC2a^+$ ratio, an indicator of cardiomyocyte maturity, is dependent on the developmental stage and stimulation regime of hiPS cell-derived cardiomyocyte. The enhancing contractile demands induced the adult-like cardiac morphology, which is important for high force generation in early-stage intensity-trained tissue. Under these conditions, tissue developed adult-like gene expression and adult-like tissue ultrastructure throughout the oxidative metabolism, tissue volume, FDAR (frequency-dependent acceleration of relaxation), positive FFR (force–frequency relationship), and physiological Ca handling.¹³² This tissue model would be useful for studies of cardiac disease and development.

5.3.2 Effect of Cell Cultivation Materials on hPS Cell Induction into Cardiomyocytes

Burridge and his colleagues examined the effect of cell cultivation biomaterials on the induction of hPS cells into cardiomyocytes using a Type E induction

protocol (Figure 5.10(f)).¹⁸ hPS cells were cultivated on plates coated with (1) human fibronectin (FN), (2) FN mimetic oligopeptides, (3) recombinant human LN (LN-511 and LN-521), (4) recombinant human VN, and (5) recombinant human E-cadherin; these ECM molecules were reported to let hPS cell adhesion and proliferation as well as to keep their pluripotent state.¹⁸ hPS cells on LN-511 and LN-521 in Essential 8 media indicated better proliferation speed in comparison to hPS cells on human FN, recombinant human E-cadherin, or recombinant human VN, probably because of the interaction between $\alpha_6\beta_1$ integrin and LN-511 or LN-521. In this research, FN-related biomaterials could not maintain the pluripotent growth of hPS cells, and only LN-related biomaterials supported long-term attachment (>two weeks) during cardiac induction. hPS cells could attach to LN-521, LN-511, and Matrigel through $\alpha_6\beta_1$ integrin, because hPS cell-derived cardiomyocyte expresses integrins β_5 , β_1 , α_V , α_7 , α_6 , α_5 , and α_3 ,¹³³ while hPS cell-derived cardiomyocyte holds lower level of $\alpha_V\beta_5$, which attaches to VN. Table 5.4 summarizes the integrin-binding sites of stem cells on some ECM proteins.¹³⁴

Single-cell real-time RT-PCR results and electrophysiological evaluation suggested that hPS cell-derived cardiomyocyte generated using this method progressed from an unspecified cardiomyocyte precursor to an immature and predominantly ventricular phenotype. The chemically defined media used in this research contribute to a reproducible and scalable protocol for the induction of hPS cells into cardiomyocyte that promotes the translation of hPS cell study into the clinical therapy of hPS cell-derived cardiomyocyte.

Wang and his colleagues investigated the preparation of functional engineered human cardiac patches using hiPS cell-derived CM, hiPS cell-derived fibroblasts (CD90⁺ cells), and decellularized rat heart ECM as scaffold.¹³⁵ The engineered human cardiac patch could be tailored to any demanded shape and size and showed normal electrical physiology and contractile *in vitro*. The decellularized rat heart ECM promoted maturation

Table 5.4 ECMs and their cell binding sites.¹³⁴ Adapted from ref. 134 with permission from American Chemical Society, Copyright 2012.

ECM	Binding site of cells
Collagen I	Integrin ($\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha V\beta 3$)
Collagen II	Integrin ($\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 10\beta 1$)
Collagen IV	Integrin ($\alpha 2\beta 1$, CD44)
Fibronectin	Integrin ($\alpha 4\beta 1$, $\alpha 5\beta 1$, $\alpha V\beta 3$, $\alpha IIb\beta 3$, $\alpha V\beta 6$, $\alpha V\beta 5$)
Laminin	Integrin ($\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 6\beta 4$)
Laminin-1 (laminin-111)	Integrin ($\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 6\beta 1$, $\alpha 7\beta 1$, $\alpha 9\beta 1$), α -dystroglycan, sulfate, and heparan sulfate proteoglycan
Laminin-5 (laminin-332)	Integrin ($\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 6\beta 4$)
Laminin-511	Integrin ($\alpha 6\beta 1$)
Laminin-521	Integrin ($\alpha 6\beta 1$)
Laminin-10/11	Integrin ($\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 6\beta 4$)
Vitronectin	Integrin ($\alpha V\beta 3$, $\alpha V\beta 5$)
(linear RGD)	Integrin ($\alpha IIb\beta 3$, $\alpha 5\beta 1$, $\alpha V\beta 3$)
(cyclic RGD)	Integrin ($\alpha IIb\beta 3$, $\alpha 5\beta 1$, $\alpha V\beta 3$)

of human iPS cell-derived CM. When the engineered human cardiac patch was located on the infarct area of the heart of rat myocardial infarction (MI) models, the engineered human cardiac patch extensively promoted to decrease the infarct size and improved the heart function of the rats after acute MI.¹³⁵ These engineered human cardiac patch may be of great value for drug screening and disease-specific heart regeneration, and meet the demands for individual-specific heart tissue for personalized regenerative therapies of myocardial damages in the future.

Fong and his colleagues evaluated the idea that native cardiac ECM and 3D cultures enhance the maturation of hiPS cell-derived CMs *in vitro*.¹³⁶ They showed that the maturation of hiPS cell-derived CMs was facilitated when the cells were inoculated into 3D cardiac ECM scaffolds, compared with 2D cultivation. 3D cardiac ECM that promoted enhanced expression of calcium-handling genes (*CASQ2*, *Triadin*, *SERCA2a*, *HCN4*, *NCX1*, *CaV1.2*, and *Junctin*).¹³⁶ They also found that hiPS cell-derived CMs in 3D adult cardiac ECM showed enhancement of kinetics (maximum upstroke and downstroke) and calcium signaling (amplitude) compared with cells in 2D cultivation. The hiPS cell-derived CMs in 3D cultivation showed more responsive signal to caffeine, which reflected an increased availability of calcium in the sarcoplasmic reticulum.¹³⁶ Therefore, these investigations also suggest the strategies for maturing hPS cell-derived CM, which may have applications in drug screening and regenerative medicine to cure heart diseases.

Effect of elasticity of cell culture biomaterials on the maturation of hPS-derived CM was evaluated by Hirata and Yamaoka.¹³⁷ They studied the effect of the cell culture biomaterials on the cardiomyocyte differentiation of mouse iPS cells *in vitro* by separately evaluation of the following continuous three steps: (a) cardiac marker gene expression, (b) self-beating and contractile gene expression, and (3) beating duration, which were compared with the results obtained from neonatal rat cardiomyocyte (NCM). hPS-derived CM and NCM were cultured on polyacrylamide hydrogels having the elastic modulus of 9, 20, and 180 kPa as well as hard TCP plates immobilized with FN, GEL, and COL type I.¹³⁷ The cardiac marker gene (*GATA4*, *Tbx5*, *MEF2C*) expression of mouse iPS cells at 14 days of differentiation was extremely high on the TCP coated with GEL or FN, whereas the undifferentiated marker gene (*Nanog*) expression of mouse iPS cells was highly supported on the hydrogels grafted with FN having 9 kPa modulus. The expression of the contractile genes *TnT2*, *TnC1*, and α -MHC and the self-beating of neonatal rat cardiomyocyte were found to be high on TCP plates coated with FN and the hydrogels immobilized COL type I.¹³⁷ The beating duration of neonatal rat cardiomyocyte was effective on the hydrogels, and mouse iPS-derived CM and neonatal rat cardiomyocyte stopped beating on the TCP plates but were still beating on the hydrogels at 21 days.¹³⁷ Overall, the cardiac differentiation from mouse iPS cells preferred rigid TCP plates coated with ECM, whereas the polyacrylamide hydrogels immobilized COL type I and having 20 and 180 kPa supported the beating-behavior (beating colony numbers and duration) of mouse iPS-derived CM and neonatal rat cardiomyocyte.¹³⁷

These results may be important for designing the cell cultivation materials for cardiac induction of hPS cells for regenerative medicine.

Horton and his colleagues examined the synergistic effects of cultivating hPS cells in hypoxia (5% oxygen) and on a cell cultivation biomaterial on the induction of hPS cells into cardiomyocyte.¹⁰⁵ They considered that the hypoxia-induced secretion of ECMs by hPS cells would facilitate the induction into cardiomyocyte. Then, they evaluated the expression of four ECMs; FN, COL I, COL IV, and LN, on EB obtained from hES cells (H9 and H1) in normoxic (21% oxygen) and hypoxic conditions. Hypoxic EB suggested higher COL I, COL IV, and FN expression in comparison to normoxic EB on days 9–13 that indicates the increased duration of mesoderm gene expression.¹⁰⁵ Moreover, the production of contracting EB increased on either COL IV, GEL, or FN under hypoxic conditions in comparison to normoxic conditions (Figure 5.15).¹⁰⁵ GEL/hypoxia cultivation synergistically enhanced the cardiomyocyte (cTnT⁺ cells) production by 5.5- and 1.7-fold relative to COL IV/normoxia and GEL/normoxia cultivation, respectively.¹⁰⁵ This research indicates that coupling ECM and hypoxia as stem cell cultivation biomaterials might mimic the physiological and dynamic environment of the stem cell niche for hPS cell induction.

An extensively chemical survey of cell cultivation biomaterials for inducing hPS cells into cardiomyocyte was not investigated. Then, Patel and his colleagues prepared around 700 biopolymers and examined these biopolymers as cell cultivation materials for hES cell-derived cardiomyocyte using a high-throughput screening method (Figure 5.16).¹⁰⁸ The best three copolymers (3/18, 15/17, and 14/17) were scaled up for extended cultivation of hES

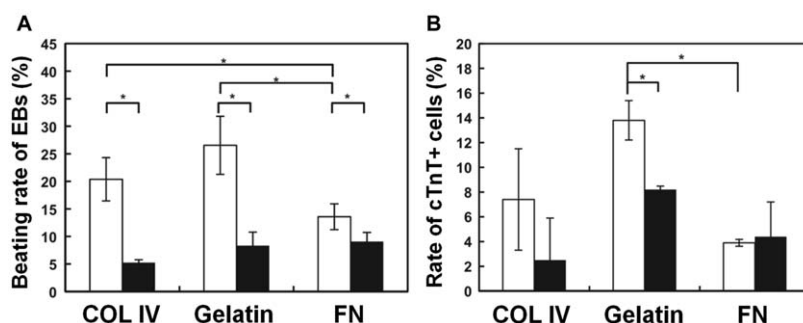


Figure 5.15 Synergistic effects of ECM cues and hypoxia on hPS cell induction into cardiomyocytes. (A) Beating rate of EBs cultivated on FN, GEL, or COL IV and exposed to either normoxic (closed bar) or hypoxic (open bar) conditions for 15 days. There was a greater number of beating EBs with exposure to hypoxia. The highest percentage of beating EBs formed when cultivation was on GEL and there was exposure to hypoxia. (B) cTnT expression by EBs cultivated on FN, GEL, or COL IV as evaluated by flow cytometry after exposure to either normoxic (closed bar) or hypoxic (open bar) conditions. Culture on GEL under hypoxic conditions formed the greatest percentage of cTnT-positive cells (14%).¹⁰⁵ Adapted from ref. 105 with permission from Elsevier, Copyright 2012.

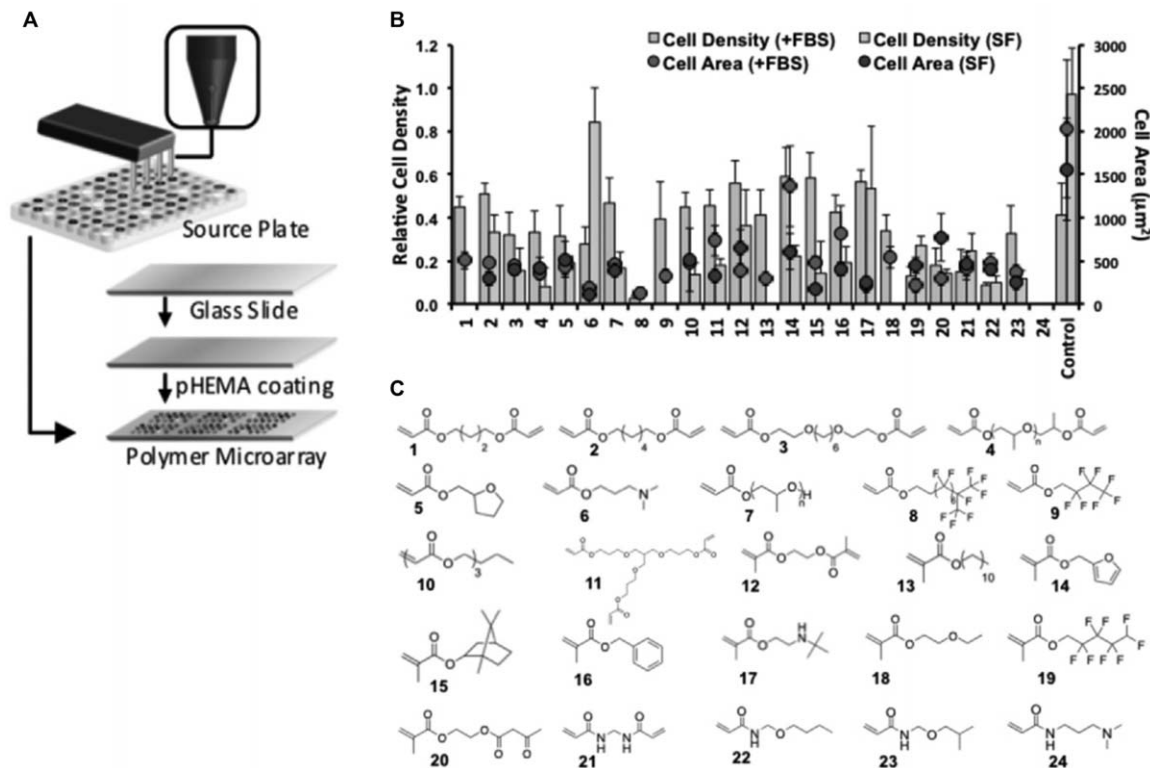


Figure 5.16 Preparation of polymer microarray and cultivation of cardiomyocytes derived from hES cells. (A) Process of polymer microarray generated by contact printing on a glass slide coated with pHEMA [poly(2-hydroxyethyl methacrylate)] to avoid background cell attachment. (B) Cell size (circle) and attachment (bar) on 24 chosen polymeric materials from an initial 116 polymer screen in FBS pre-treated conditions (left) and serum-free (SF, right). (E) Monomers used for the polymeric microarrays described in (B).¹⁰⁸

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cell-CMs for 15 days and were evaluated using a patch clamp method and myofibril analysis; the structural and functional phenotypes were kept on the synthetic biomaterials with no ECM as coating materials. The co-biopolymers of 3/18, 15/17, and 14/17 maintained larger cell size (899 μm^2 , 1033 μm^2 , and 977 μm^2 , respectively) and high cardiomyocyte densities (Figure 5.17A).¹⁰⁸

Cardiomyocytes were evaluated for α -actinin staining as a rapid gauge of structural integrity. The distance between the Z bands indicates sarcomere length, the basic motor units that make up the myofibril (Figure 5.17C).¹⁰⁸ Relative to control hES cell-cardiomyocyte on GEL (sarcomere length = 1.50 μm), the sarcomere length on co-biopolymers 3/18, 15/17,

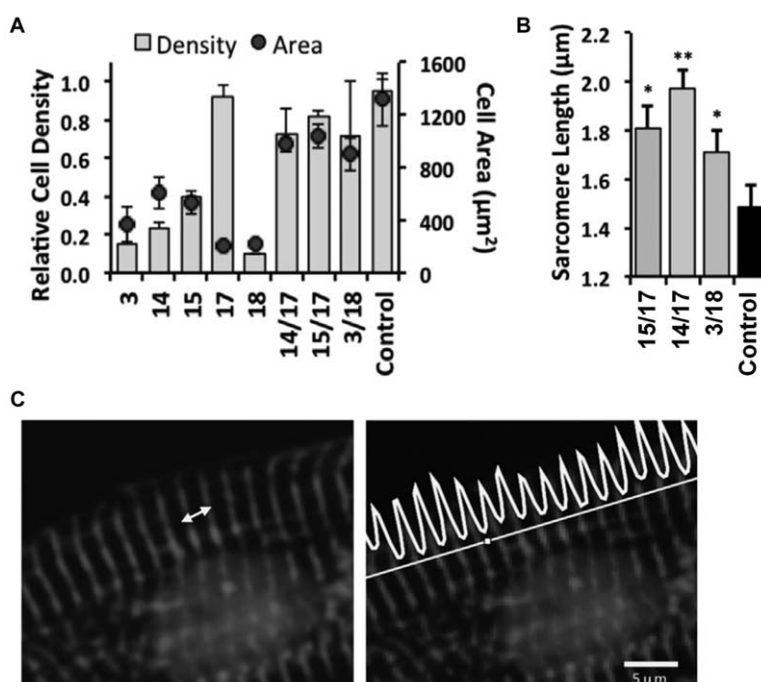


Figure 5.17 Characterization of cardiomyocytes derived from hES cells after 15 days of cultivation on polymers, copolymers, or 0.1% GEL-coated plates (control). (A) Cardiomyocyte cell size (circle) and attachment (bar) on chosen copolymers (14/17, 15/17, and 3/18) in comparison to their constituent homopolymers (3, 14, 15, 17, and 18). Monomer structure of polymers are shown in Fig. 5.16. (B) The length of each sarcomere in cardiomyocytes derived from hES cells, which were cultivated on 0.1% GEL-coated plates (control) and copolymer surface. Structural maturity was enhanced as shown by longer sarcomere length in cardiomyocytes cultivated on synthetic copolymer surface in comparison to control. (C) Structural analysis of cardiomyocytes. The white arrow shows a sarcomere unit. The length of each sarcomere was evaluated from the Image J line profile tool.¹⁰⁸

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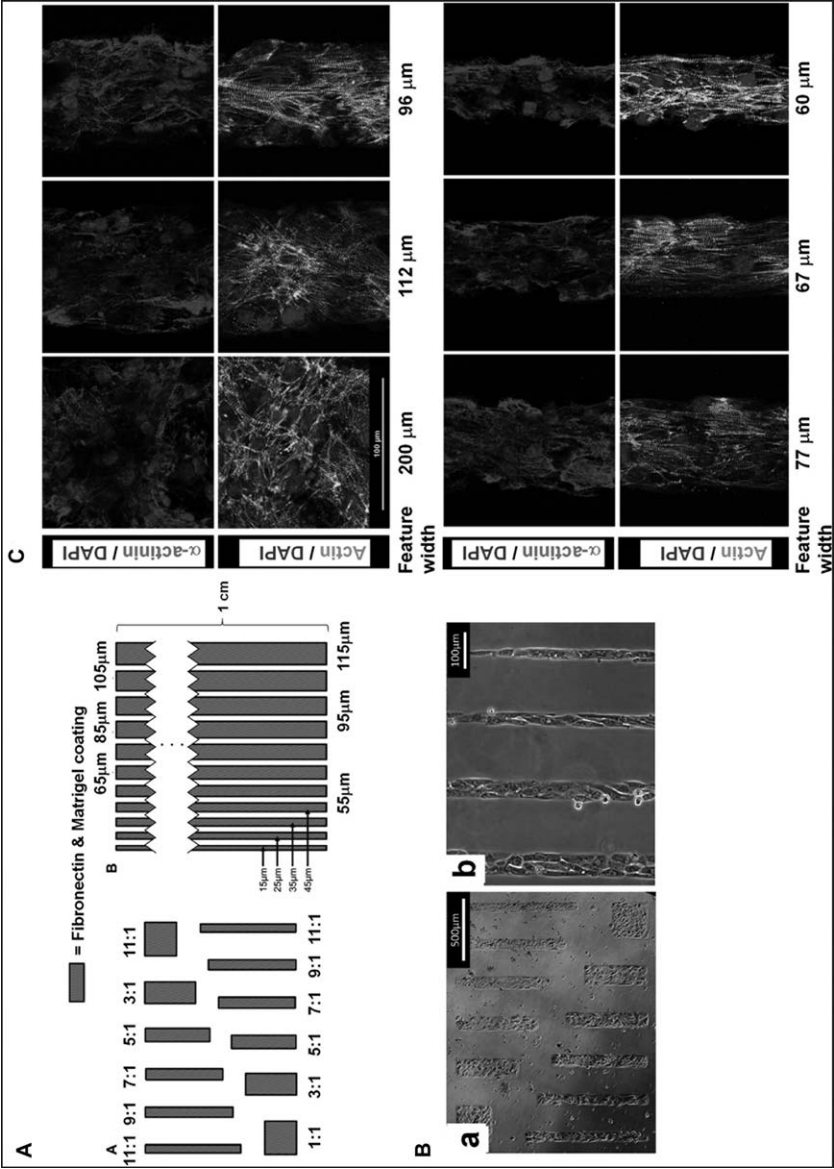
and 14/17 were significantly longer (1.70 μm , 1.80 μm , and 1.97 μm , respectively) (Figure 5.17B).¹⁰⁸ Structural maturity was enhanced, as detected by the longer sarcomere length of cardiomyocyte cultivated on synthetic biopolymers in comparison to the control GEL-coated surface. The chemical moieties detected in this high-throughput screen indicate chemically defined conditions for the manipulation and cultivation of hES cell-derived cardiomyocyte and build up framework for the promising design of distinct synthetic materials.

hPS cell-derived cardiomyocytes usually generate monolayers of contracting cells and/or randomly-aligned clusters that cannot mimic the barrel-shaped, aligned, and organized cells, which are found in the natural adult myocardium.^{95,138} Moreover, biochemical response properties, multinucleation, MHC (myosin heavy chain) isoform expression, and Ca^{2+} handling, which are generally observed in mature adult cardiomyocyte are not shown by immature hPS cell-derived cardiomyocyte.^{95,139} Typically, long cell cultivation (2–4 months) of immature hPS cell-derived cardiomyocyte makes a more mature cardiomyocyte phenotype.⁹⁵ However, the hPS cell-derived cardiomyocyte still do not show the level of sarcomere alignment and structure found in natural adult cardiomyocyte, although hPS cell-derived cardiomyocyte cultivated for long time exhibits better sarcomere alignment.^{95,140}

2D micropatterning investigation indicated that the multi-cell micropattern gives a key function in regulating the sarcomere morphologies of cardiomyocyte derived hPS cells. The micropatterned cultivation of hPS cell-derived cardiomyocyte with widths between 80 μm and 30 μm promoted excellent cell alignment with an extensive increase of sarcomere alignment, which is relative to the long axis of the micropatterns (Figure 5.18).⁹⁵ The generation of extensively aligned cardiomyocyte clusters with robust sarcomere structures indicates the important potential for the clinical therapy of hPS cell-derived cardiomyocytes and advancing cell-based pharmacological investigations.

Ribeiro and his colleagues induced hES cells (H7) into cardiomyocytes using the method reported by Lian and his colleagues¹⁷ to investigate the effects of cell culture substrate stiffness and cell shape on sarcomere activity and function.⁹⁷ hES cell-derived cardiomyocyte was cultivated on Matrigel-patterned polyacrylamide hydrogel with aspect ratios of 1:1, 3:1, 5:1, and 7:1 on 2000 μm^2 rectangles where the polyacrylamide hydrogel was made with the physiological stiffness of healthy myocardium (10 kPa).⁹⁷

Sarcomeres laterally registered with sarcomeres in neighboring myofibrils in hPS cell-derived cardiomyocyte cultivated on the rectangular surface with an aspect ratio of greater than 3:1, while myofibrils randomly oriented on square-patterned or unpatterned surfaces.⁹⁷ Moreover, the contractile force enhanced with increasing of the cell aspect ratio. hES cell-derived cardiomyocyte was also cultivated on Matrigel-micropatterned hydrogel with the stiffness of fibrotic or ischemic myocardium (35 kPa) or the stiffness of embryonic myocardium (6 kPa). Enhancement of cell cultivation material stiffness generates myofibril defects on the material with an aspect ratio of 7:1.⁹⁷



These findings indicate that the physiological stiffness of the cell cultivation material (10 kPa) and cell shape regulate the distribution of intracellular tension, which is important for sarcomere contractility and activity of cardiomyocyte derived from hPS cells. Single-cell cultivation of hES cell-derived cardiomyocyte would offer an important design for investigating the cause of myocardial contractility and heart disease at the cellular level.

Some other attractive materials have also been used for the cultivation and induction of hPS cells into cardiomyocytes.^{96,110,117,118} A large number of hPS cell-derived differentiated cells is demanded in clinical therapies. Therefore, a scalable platform using polymeric microcarriers for the differentiation and cultivation of hPS cells would supply a readily available stock of induced cells, *e.g.*, cardiomyocytes, for cardiotoxicity tests, drug screening, and cell therapy. The suspended microcarrier cultivation is a valuable protocol for culture of hPS cell-derived differentiated cells.

Lecina and his colleagues examined the generation of hES cell-derived cardiomyocyte for optimization of the microcarrier cultivation system using the Type E induction protocol.⁹⁶ Some LN-coated microcarriers (DE-53, Cytodex-3, Cytodex-1, FACT, and TOSOH-10) were used to evaluate the effects of microcarrier shape, size, type, and cell density on the efficiency of inducing hPS cells into cardiomyocyte. Microcarrier size was found to be the most affecting factor, which gave the effect on size distribution and clustering morphologies of hES cells. Protamine-coated TOSOH-10 microcarriers (10 μm diameter) formed smaller clusters of hES cells surrounded by the microcarriers. DE-53 microcarriers (positively charged; cylindrical cellulose; length 150–400 μm ; diameter 35–50 μm) was found to result in compact cell-microcarrier clustering of hES cells. Bigger microcarriers, such as Cytodex-3 (crosslinked dextran; diameter 140–215 μm), Cytodex-1 (crosslinked dextran; diameter 140–260 μm), and FACT (crosslinked polystyrene; diameter 125–215 μm), generated a broader size distribution and bigger clustering of hES cells.⁹⁶ However, different surface properties, such as the denatured COL type I of FACT and Cytodex-3 or the *N,N*-diethylaminoethyl group (tertiary amine) of Cytodex-1 did not contribute to aggregate morphology and the size

Figure 5.18 Sarcomere development by cardiomyocytes derived from hES cells on micropattern surfaces. (A) Micropattern scheme. ECMs were micropatterned into rectangles of varying size and aspect ratio. Two kinds of patterning were used. (B) Representative bright-field pictures of cardiomyocytes derived from hES cells on micropatterned surfaces. Cells were cultivated on micropatterned Matrigel-FN areas adjusted to the printed morphologies. (C) Actin, α -actinin, and DAPI staining of cardiomyocytes after 5 days in cultivation on micropatterned surfaces with different widths. Myofilament markers (α -actinin and actin) showed superior sarcomere organization, sarcomere alignment, and cellular alignment on micropatterned surfaces less than 100 μm in width. The cells cultivated onto a square micropatterned surface (200 μm in this set) did not exhibit any defined alignment.⁹⁵

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distribution of hES cells. hES cells cultivated on soft TOSOH-10 microcarrier (protamine-derivatized 10 μm beads) formed 90% beating clusters, while hES cells on the stiffer microcarrier formed a lower percentage of beating clusters (for example, 58–62% for DE-53).⁹⁶ hES cell-derived cardiomyocyte was formed at 19–21% on TOSOH-10 microcarrier and at 10–16% on other microcarriers examined in this research, which were analyzed by flow cytometry for expression of α -actinin and MHC.⁹⁶ This investigation suggests a scalable bioprocessing system for production of hPS cell-derived cardiomyocytes using polymeric microcarriers, which comply with GMP.

Self-assembled oligopeptide amphiphile molecules were further used as cultivation materials for hPS cell-derived cardiomyocyte. Ikonen and his colleagues cultivated hES cell-derived cardiomyocyte in 3D and 2D self-assembling nanofibrous hydrogel (Figure 5.19).¹¹⁸ hES cell-derived cardiomyocyte cultivated on appropriate self-assembling nanofibers co-assembled with HyA were found to be extensively cultivated for a long time. hPS cell-derived cardiomyocyte encapsulated in injectable hydrogels, consisted of self-assembled oligopeptide amphiphiles¹¹⁸ or alginate,¹¹⁰ should be attractive for future stem cell therapies in cardiac regeneration.

5.4 Induction into Hepatocytes

Some excellent protocols for inducing hES cells and hiPS cells into hepatocyte are proposed currently. Then, we evaluate the currently proposed protocols for the sufficient induction of hPS cells into hepatocyte and the effect of cell cultivation materials on these processes.^{141–149} Several researches on hepatocyte induction are contained in Table 5.5,^{141–151} and several research of the corresponding methods with timelines are depicted in Figure 5.20.

5.4.1 Efficient Protocols for hPS Cell Induction into Hepatocytes on Materials

Yamazoe and his colleagues examined the hepatic induction of hPS cells (201B7, Toe, and KhES3) cultivated on commercially available polyamide electrospinning nanofiber (280 nm diameter, Ultra-Web) using the Type E induction protocol,¹⁴¹ which is similar to the protocol developed by Ramasamy and his colleagues (Figure 5.20(b))¹⁴² or Farzaneh and his colleagues (Figure 5.20(a)).¹⁴³ Yamazoe and his colleagues compared the function and differentiation efficiency of hPS cells cultivated on the nanofiber and on several other cell culture materials (TCP dishes coated with COL type 1, GEL, CellStart or Matrigel). ES cells were differentiated into hepatocytes on Ultra-Web nanofibers coated with Matrigel and on other ECM-coated dishes (*e.g.*, TCP dishes covered with GEL, Matrigel, CellStart, or COL type I), and functional hepatocyte analysis were performed to investigate the effect of cell cultivation material on the induction of ES cells into hepatocyte. ES cells cultivated on nanofiber displayed higher hepatic function than ES cells

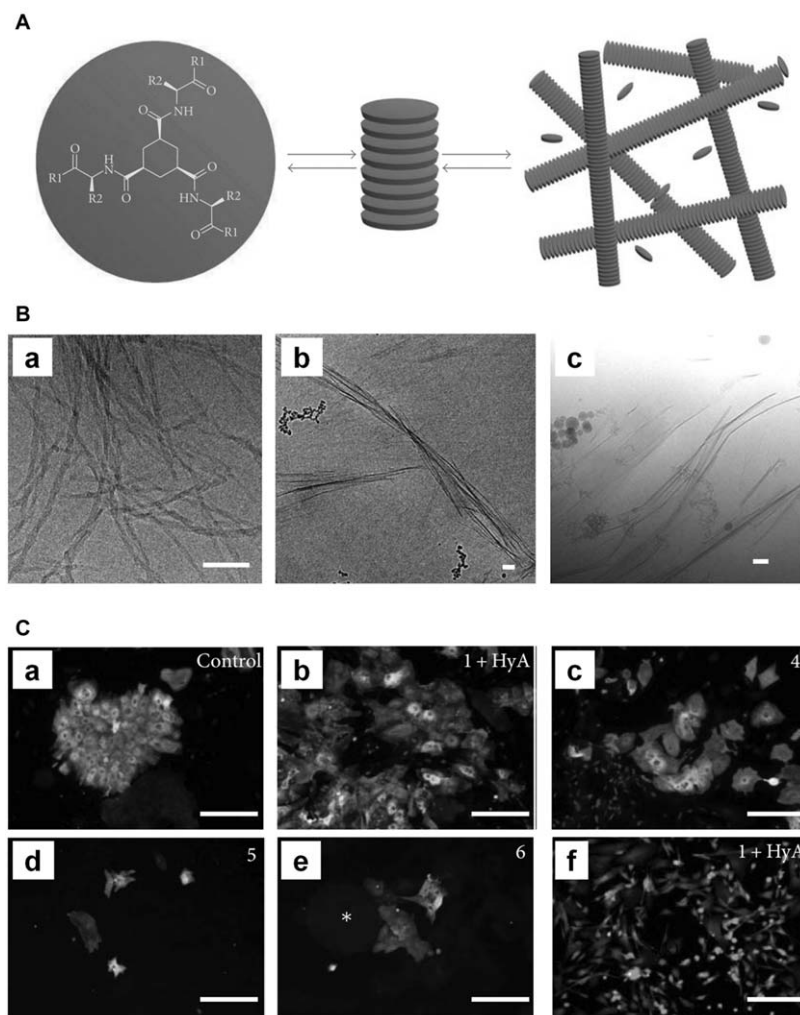


Figure 5.19 Cardiomyocytes derived from hES cells in self-assembled nanofiber hydrogels. (A) A schematic of the generation of nanofibers from peptide amphiphile molecules *via* self-assembly. (B) Cryo-TEM (transmission electron microscopy) pictures of nanofiber hydrogels 3 [0.03 wt% (a)], 4 [0.2 wt%, (b)], and 5 [0.05 wt%, (c)]. (C) Cardiomyocytes derived from hES cells on several nanofiber coatings stained with cardiac antibodies ((a)–(e)) and a LIVE/DEAD kit (f). cTnT expression is described in (a) to (e). MLC2v expression is also shown in (a). MHC expression is also shown in (b) to (e). DAPI counterstaining was used to observe cell nuclei ((a) to (e)). Cardiomyocytes derived from hES cells were cultivated on GEL-coated plates [control, (a)], nanofiber hydrogel 1 co-assembled with HyA (b), nanofiber hydrogel 4 (c), nanofiber hydrogel 5 (d), or nanofiber hydrogel 6 (e). The chemical schemes of the peptide amphiphile molecules of hydrogels 1, 3, 4, 5, and 6 were previously described.¹¹⁸ Adapted from ref. 118, <http://dx.doi.org/10.1155/2013/285678>, under the terms of the CC BY 3.0 license, <https://creativecommons.org/licenses/by/3.0/>.

Table 5.5 Differentiation of hPS cells into hepatocytes cultured on biomaterials.⁵ Adapted from ref. 5 with permission from Elsevier, Copyright 2016.^a

hPSCs	Cell culture materials	Methods	Cell type (%)	Ref. (year)
hESCs (UCO6), hiPSCs (iPSW)	Collagen-coated dishes	Type B	Hepatocytes	151 (2016)
hESCs (Royan H5)	Electrospun polyamide (Ultra-Web) coated with Matrigel	Type E	Hepatocytes	143 (2010)
hESCs (SA002)	Matrigel-coated hollow fiber bioreactor	Type E	Hepatocytes (ASGPR1, 29%)	147 (2011)
hESCs (KhES3), hiPSCs (Toe, 201B7)	Polyamide nanofibers (Ultra-Web) ($d = 280$ nm) coated with Matrigel	Type E	Hepatocytes	141 (2013)
hESCs (H1)	Porous alginate (Algimatrix) scaffolds	Type E	Hepatocytes	142 (2013)
hESCs (H9)	Matrigel-coated microcarriers (Cytodex 3)	Type E	Hepatocytes (ASGPR1, 17%)	144 (2014)
hiPSCs	Chitin, alginate ion-complex hydrogel fibers containing galactose and collagen	Type E	Hepatocytes (AFP ⁺ cells, 43%; albumin ⁺ cells, 48%)	148 (2014)
hESCs (H9)	Matrigel-coated dishes	Type E	Hepatocytes	149 (2015)
Porcine iPSCs	Decellularized liver ECM, Matrigel and collagen-coated dishes	Type E	Hepatocytes	163 (2016)
hESCs (H7, H9), hiPS (IMR90)	Laminin-511, Laminin-521, or fibronectin-coated dishes	Type E	Hepatocytes	160 (2016)
Liver progenitor (HepaRG), hESC-derived hepatocytes	PEG/HA IPN hydrogels	Type E	Hepatocytes	159 (2017)
hESCs (H1)	Matrigel-coated dishes	Type E	Hepatocytes	150 (2017)
hESCs (H9), hiPSCs (201B7, Tic)	Matrigel-coated dishes	Type F and Type H	Hepatocytes	146 (2012)
hESCs (H1, H9), hiPSCs (Tic, Toe, Dotcom)	Matrigel, nanopillar plate	Type E and Type H	Hepatocytes	145 (2013)

^aHA, hyaluronic acid; IPN, interpenetrating polymer network; PEG, polyethylene glycol.

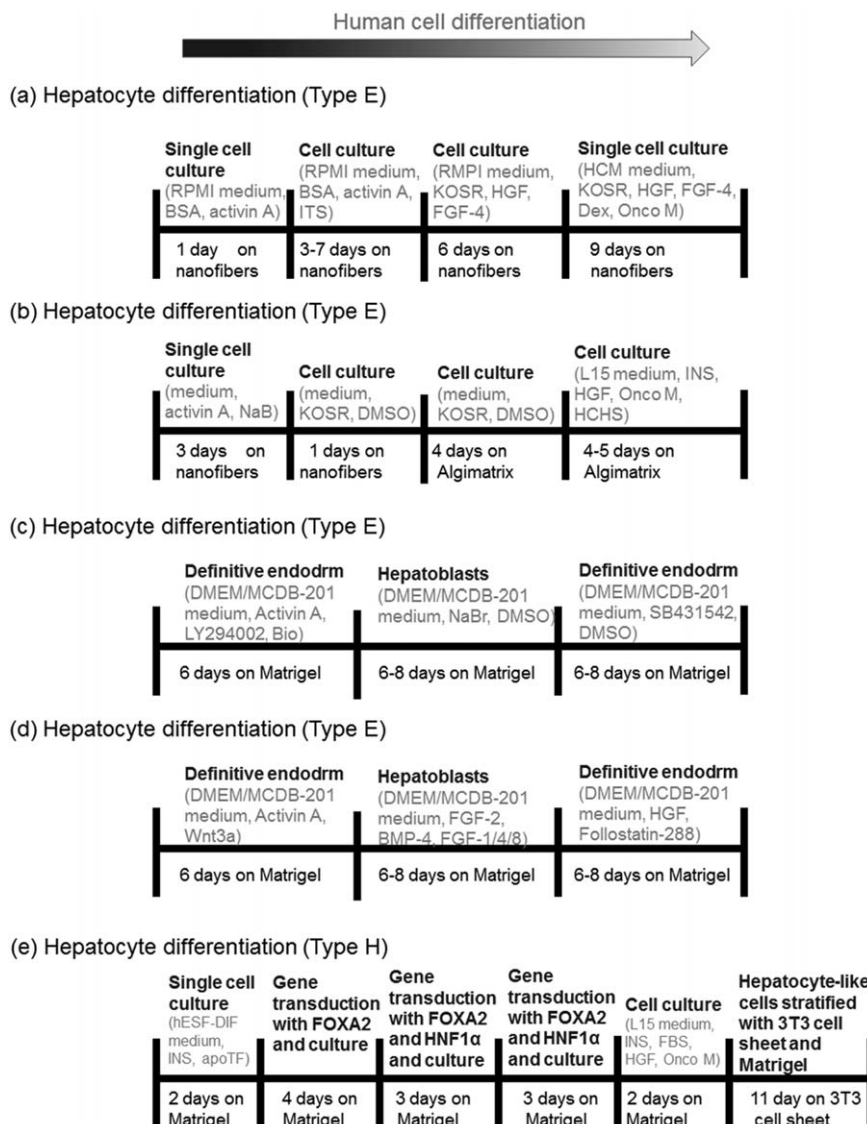


Figure 5.20 Timelines of protocols for differentiation of hPS cells into hepatocytes: (a) the Type E induction protocol studied by Farzaneh and his colleagues.¹⁴³ (b) The Type E induction protocol studied by Ramasamy and his colleagues.^{5,142} (c) The Type E induction protocol using small molecules studied by Tasnim and his colleagues.^{5,149} (d) The Type E induction protocol using growth factors studied by Tasnim and his colleagues.^{5,149} (e) The Type H induction protocol studied by Nagamoto and his colleagues.^{5,146} Adapted from ref. 5 with permission from Elsevier, Copyright 2016.

cultivated on other ECM-coated dishes.¹⁴¹ The nanofiber looks like recapitulating the stem cell niche that regulates the induction of hPS cells into desired cell lineages.

A scalable reactor system is important to provide large amounts of induced cells, such as hepatocytes derived from hPS cells. The microcarrier cultivation condition has a large surface to volume ratio and, then, increases the cell cultivation in a bioreactor system efficiently. Park and his colleagues studied a microcarrier cultivation system for the directed induction of hPS cells into hepatocyte using GEL microcarriers (CultiSpher), COL microcarriers (SphereCol), and crosslinked dextran microbeads (Cytodex 3 and Cytodex 1) coated with Matrigel.¹⁴⁴ hES cells (H9) were induced into hepatocyte on microcarrier using the method reported by Roelandt and his colleagues,¹⁵² which is a Type E induction protocol where hES cells on microcarrier were cultivated in serum-free basal induction media composed of Dulbecco's Modified Eagle Medium (DMEM) and MCDB-201 included with beta-mercaptoethanol, dexamethasone, insulin-transferrin-selenium (ITS), linoleic acid BSA (LA-BSA), and ascorbic acid 3-phosphate. The following growth factors were included in the basal media: (a) day 0: Wnt3a and Activin A; (b) day 2: Activin A; (c) day 4: BMP4; (d) day 8: FGF-1; and (e) day 12: HGF.

Hepatic gene expressed higher in hES cell-derived hepatocyte cultivated on Cytodex 1 and Cytodex 3 (dextran-based microcarriers) than hepatic genes expressed in those cultivated on CultiSpher (GEL-based) or SphereCol (COL) microcarriers (Figure 5.21).¹⁴⁴ hES cell-derived hepatocyte cultivated on Cytodex-3 in a stirred bioreactor or in static cultivation showed the expression of both mature and immature hepatocyte-lineage proteins and genes, such as albumin and ASGPR-1 (asialoglycoprotein receptor-1) (Figure 5.21). hES cell-derived hepatocyte expressed some functional hepatic characteristics, such as urea and albumin secretion as well as CYP3A4 activities.¹⁴⁴

One of the demerits of hPS cell cultivation and induction on microcarriers is the low initial adhesion ratio of hPS cells on the microcarriers (typically <20%). The immobilization of high cell-binding nanosegments on the microcarrier might resolve this problem. However, microcarrier cultivation systems provide a potential system for the large-scale production of hES cell-derived hepatocyte in a tunable bioreactor system.

Hepatocytes derived from hPS cells have many hepatocyte properties, such as glycogen storage, the ability to uptake low-density lipoprotein and indocyanine green (ICG), and urea and albumin synthesis. However, the hepatocytes derived from hPS cells need further maturation to retain drug metabolism abilities; for this reason, primary hepatocytes are typically used in drug toxicity evaluation currently. Then, Takayama and his colleagues developed mature hepatocytes derived from hPS cells using a Type H induction protocol that used stage-specific transient overexpression of hepatic transcription factors (HNF1 α - and FOXA2-expressing AD vector) and a 3D spheroid cultivation condition using a nanopillar plate (Figure 5.22).¹⁴⁵

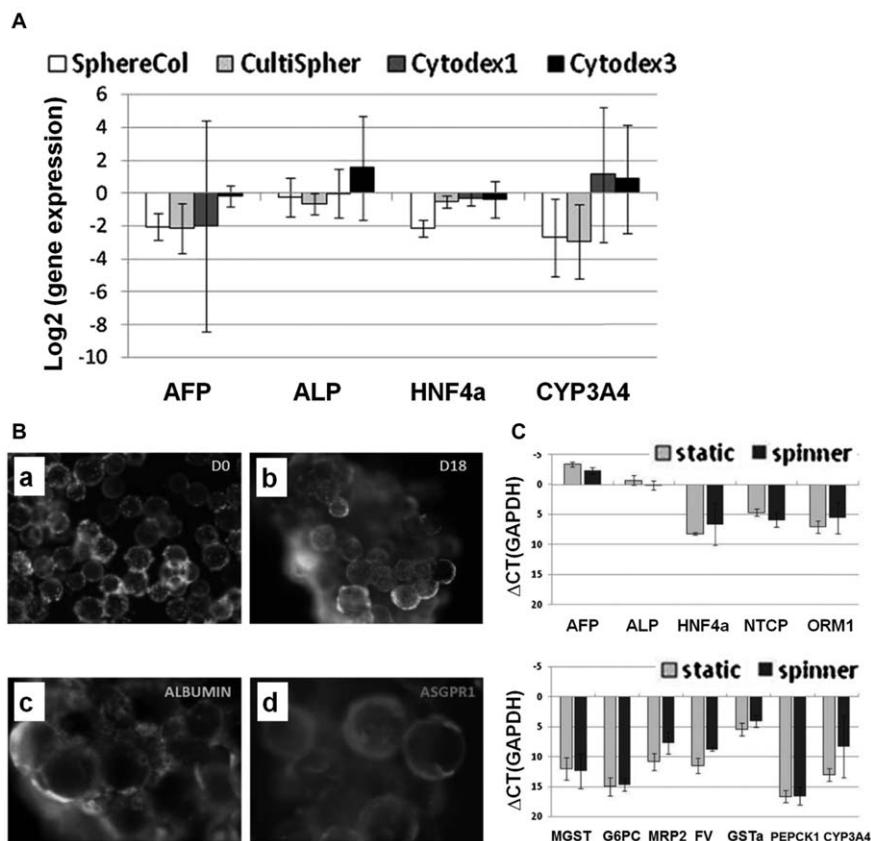


Figure 5.21 Hepatic induction of hES cells on microcarriers in spinner flask suspension cultivation. (A) Hepatic gene expression by cells differentiated on some types of microcarriers (Cytodex 3, Cytodex 1, Cultispher, and SphereCol) in static cultivation for 18 days. (B) Live-dead staining at induction day 0 (a) and day 18 (b). Immunofluorescent staining for albumin (c) and ASGPR1 (d) at day 18. (C) Hepatic gene expression in cells induced in spinner microcarrier cultivation in comparison to static microcarrier induction.¹⁴⁴

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Their designed protocol contributed to the generation of more matured hepatocytes derived from hPS cells (3D cultivation) than the hepatocytes developed using previous protocol using 2D cultivation system. The cytochrome P450 activity (CYP3A4 and CYP2C9) of hiPS cell-derived hepatocytes was higher using their protocol than the typical 2D cultivation protocol (Figure 5.22C).¹⁴⁵ Their hepatocytes derived from hPS cells secreted more metabolites in response to chemical stimulation using rifampicin. Furthermore, cytochrome P450 activity was inhibited by CYP3A4 and CYP2C9 inhibitor molecules (ketoconazole and sulfaphenazole, respectively) (Figure 5.22D).¹⁴⁵ The findings suggested that the drug metabolism abilities

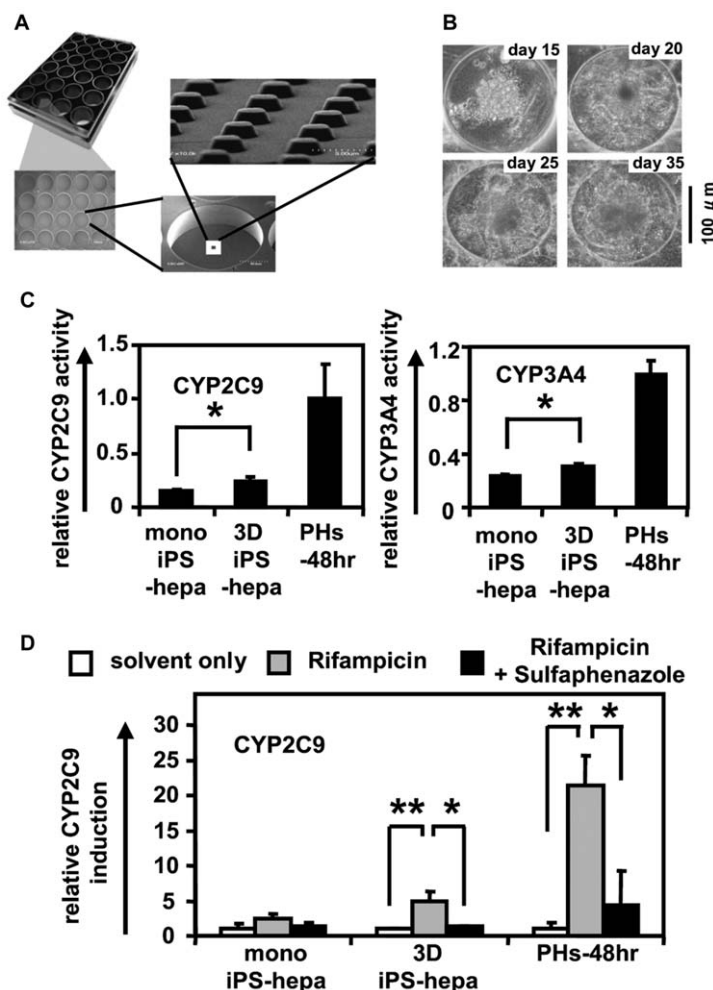


Figure 5.22 Hepatic induction of hiPS cells cultivated on nanopillar plates. (A) Picture of a 24-well nanopillar plate and the microstructure of the pillar and hole configuration. (B) Phase contrast pictures of hES cell-derived hepatocyte spheroid on the nanopillar plates. Scale bar = 100 μ m. (C) CYP activity analyzed in the monolayer cultivation of hepatocytes derived from iPS cells (mono iPS-hepa) at day 20, the nanopillar plate cultivation of hepatocytes derived from iPS cells (3D iPS-hepa) at day 35, and the cultivation of primary hepatocytes at 2 days (PHs-48 hr). On the y-axis, the CYP activity in PHs-48 hr was set to 1.0. (D) Induction of CYP2C9 by DMSO (solvent only; white bar), rifampicin (gray bar), or rifampicin plus CYP inhibitor (sulfaphenazole, black bar) in PHs-48 hr, 3D iPS-hepa, and mono iPS-hepa. On the y-axis, the CYP activity of cells cultivated in media including DMSO was set to 1.0.¹⁴⁵

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of hepatocytes derived from hPS cells produced using their 3D cultivation protocol was superior to the drug metabolism abilities of those generated in a typical 2D cultivation protocol.

Their induction method should be valuable to obtain more mature hepatocytes derived from hPS cells, although the protocol needs stage-specific transient transduction of a HNF1 α - and FOXA2-expressing AD vector as well as 3D spheroid cultivation.

Extensive energies are used for inducing hPS cells into efficient hepatocytes, which can be used for drug screening, disease modeling, and cell therapy. Typical previous methods used growth factors to recapitulate the induction signals for hepatocyte under *in vitro* conditions that are expensive protocols for generating hepatocyte from hPS cells. The use of small molecules but not growth factors in the induction of hepatocyte from hPS cells might be an attractive alternative. This is because small molecules are inexpensive and cell-permeable molecules in comparison to growth factors. Then, Tasnim and his colleagues examined a method for hES cell induction into hepatocytes using a predominantly small molecule-based protocol (SM-Heps).¹⁴⁹ The three step induction design (Figure 5.20(c)) includes the usage of appropriate concentrations of (a) BIO (bromo-indirubin-3'-oxime) and LY294002 for the induction of a definitive endoderm (DE), (b) DMSO (dimethyl sulfoxide) and sodium butyrate for the preparation of hepatoblasts, and (c) SB431542 for the induction into hepatocyte. Activin A is the only growth factor utilized in this method. The findings indicated that SM-Heps were functionally and morphologically similar to or better than hepatocyte obtained by growth factor-induced differentiation (GF-Heps) (Figure 5.20(d)) in terms of cytochrome P450 (CYP3A4 and CYP1A2) activities, urea and albumin production, and hepatic marker expression (Figure 5.23).¹⁴⁹ Cell viability evaluation after treatment with hepatotoxics (e.g., acetaminophen, diclofenac, digoxin, and troglitazone) indicated that the sensitivity of SM-Heps to these toxic molecules extensively resembles to that of human primary hepatocyte. The preparation of SM-Heps results in an 81% and 67% cost decrease in comparison to human primary hepatocytes and GF-Heps, respectively.¹⁴⁹ Then, SM-Heps would be a cost-effective and robust replacement for human primary hepatocyte for drug screening and development.

5.4.2 3D Cultivation Facilitates the Induction of hPS Cells into Hepatocytes

Hepatocyte prepared from hPS cells represents a potential unlimited cell resource for the cell therapies of damaged liver. However, the present methods for the induction of hPS cells into mature and functional hepatocyte are not sufficient. Some investigators hypothesized that mature hepatocyte could be formed from hPS cells when hPS cells were cultivated in a 3D system, which mimics the native stem cell niche. Then, Farzaneh and his colleagues considered that hPS cells may effectively be induced into mature

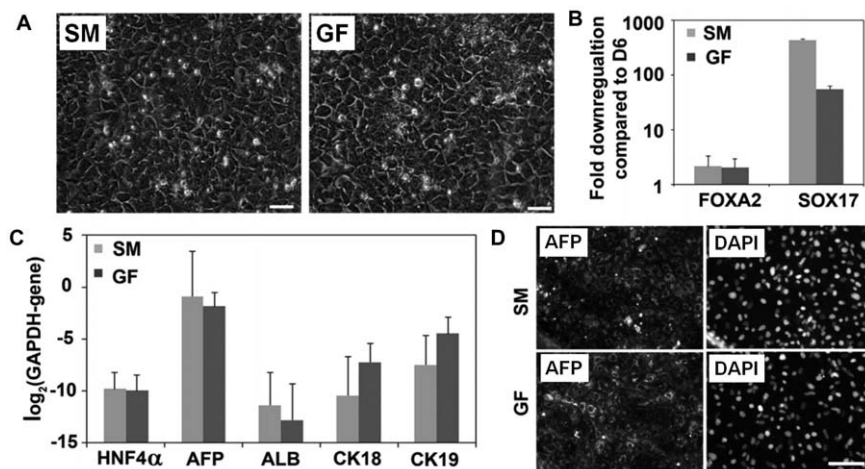


Figure 5.23 Small molecules successively induce the generation of hepatoblasts from DE. (A) Phase contrast pictures showing cell morphology in the DE phase differentiated for 8 days with growth factors (right panel) and for an additional 6 days with small molecules (left panel). Scale bar = 25 μ m. (B) qPCR assay showing no down-regulation of FOXA2 and down-regulation of the DE marker SOX17 in cells supplemented with growth factors (dark gray bars) or small molecules (light gray bars). Expression intensities of FOXA2 and SOX17 are shown relative to day 6. (C) qPCR assay showing the hepatic markers CK18, HNF4a, AFP, ALB, and HNF4a and the biliary marker CK19, showing the formation of hepatoblasts from cells supplemented with small molecules (light gray bars) or growth factors (dark gray bars). (D) AFP expression evaluated by immunofluorescence in hepatoblasts prepared using small molecules (upper panel) compared to growth factors (control; lower panel). Cell nuclei were stained with DAPI. Scale bar = 100 μ m. SM: DE induced into hepatoblasts using sodium butyrate and DMSO; GF: DE differentiated into hepatoblasts using BMP-4 and FGF-1/2/4/8.¹⁴⁹ Adapted from ref. 149 with permission from Elsevier, Copyright 2011.

hepatocyte when hPS cells were differentiated on 3D nanofiber surface using commercial Ultra-Web polyamide electrospinning nanofibers with the Type E induction protocol (Figure 5.20(a)).¹⁴³ They observed that hepatic lineage markers expressed on hPS cell-derived hepatocyte during the step-wise induction steps, and functional mature hepatocyte was finally generated. Hepatocytes derived from hES cells on Matrigel-coated Ultra-Web nanofibers exhibited higher urea generation and higher glycogen storage as well as lower secretion of AFP (α -fetoprotein). Moreover, the hepatocytes showed the expression of organic anion ICG and exhibited LDL (low-density lipoprotein) uptake and metabolic PROD activity, which are properties of mature hepatocyte. These findings suggest that a topographically controlled cell culture material at the nano level is able to regulate the induction of hES cells into hepatocyte with distinct mature functionality, which might be used for stem cell therapy.

Co-cultivation methods under 3D cultivation system can keep the primary hepatocyte functions.^{153–155} For example, Kim and his colleagues observed that primary rat hepatocyte could keep hepatocyte functions in 3D co-cultivation on endothelial cell sheets, which were made by cultivation of the cells on a temperature-responsive poly(*N*-isopropylacrylamide)-grafted material.¹⁵⁴ Moreover, hPS cells can effectively induce into hepatocytes when HNF1 α (hepatocyte nuclear factor 1 homeobox A) and FOXA2 (forkhead box A2) genes are transduced into hPS cells. Then, Nagamoto and his colleagues examined the hepatic maturation of hES cell- or hiPS cell-derived hepatocytes in 3D co-cultivation system where the hepatocytes were cultivated on a fibroblast (Swiss 3T3) cell sheet using the Type H induction protocol.¹⁴⁶ Nagamoto and his colleagues generated hepatocytes derived from hPS cells using the following protocol (Figure 5.20(e)).¹⁴⁶ (a) for mesendoderm induction, hPS cells were cultivated on Matrigel-coated plates in media supplemented with FGF-2, Activin A, BSA, sodium selenite, apotransferrin and insulin for 48 h; (b) for DE induction, the mesendoderm cells were transduced with FOXA2 and HNF1 α for 1.5 h on day 6 and cultivated for another 72 h on Matrigel-coated plates in media including FGF-4 and BMP-4; (c) for the proliferation of hepatoblasts, DE cells were again transduced with FOXA2 and HNF1 α for 1.5 h and cultivated for 72 h on Matrigel-covered plates in media containing FGF-10, FGF-4, FGF-1, and HGF; and (d) for hepatocyte maturation, the cells were cultivated on Matrigel-covered plates for 48 h in media including dexamethasone, oncostatin M, HGF, insulin, FBS, and tryptose phosphate broth. Hepatocytes derived from hPS cells were cultivated on fibroblast (Swiss 3T3) cell sheets on day 14 (Figure 5.24),¹⁴⁶ and Matrigel was placed onto hepatocytes derived from hPS cells from day 15 until day 25.

Hepatocytes derived from hPS cells were extensively generated from the evaluation of the high albumin secretion and the gene upregulation of hepatocyte-related markers (cytochrome P450 enzymes); the hepatocyte properties of the cells induced using the 3D co-cultivation protocol with fibroblast sheets were found to be better than those of hepatocytes derived from hPS cells, which were prepared in 2D monolayer cultivation system. The COL type I secreted by the fibroblasts seems to play a key function in the maturation of hepatocytes.¹⁴⁶ The hepatocytes derived from hPS cells generated using the above 3D co-cultivation system with human fibroblast sheets might be valuable in medical and/or clinical uses, such as drug screening.

Ramasamy and his colleagues examined whether transferring DE cells originally generated from hES cells on 2D cultivation system into a 3D cultivation system might enhance the maturation and/or functionality of hepatocytes derived from hES cells.¹⁴² Then, Ramasamy and his colleagues cultivated hES cells (H1) on 2D plates in stage I medium using the Type E induction protocol. Subsequently, the cells were cultivated on Algimatrix scaffolds (alginate sponges) for 3D cultivation in stage II medium for few days, following cell cultivation on the alginate matrices in stage III medium

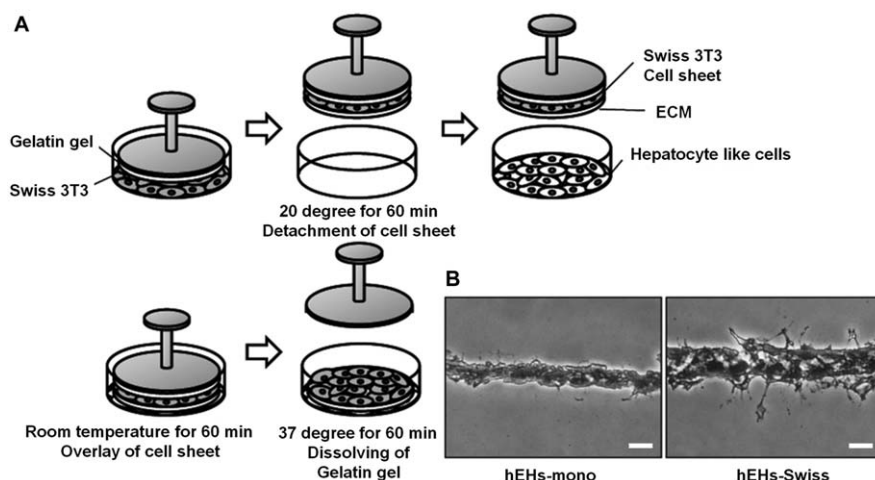


Figure 5.24 Preparation of hepatocytes derived from hPS cells on a fibroblast (Swiss 3T3) cell sheet. (A) Fibroblast was cultured on a temperature-responsive culture plate (poly-*N*-isopropylacrylamide-grafted plate). When the fibroblast became confluent, a GEL-coated cell sheet manipulator was introduced, and the cultivation temperature was decreased to 20 °C for 1 h to detach the manipulator from the cultivation plate. The fibroblast was then harvested as a contiguous cell sheet adhered on the GEL. The fibroblast sheet was then located on hiPS cell- or hES cell-derived hepatocytes (hiPHs or hEHs, respectively). The cultivation plate with the manipulator was incubated at room temperature for 1 h to form an attachment between the hiPHs or hEHs and the fibroblast sheet. To dissolve the GEL, the cultivation plate was incubated at 37 °C for 1 h. (B) Phase contrast micrographs of vertical sections of hepatocytes coated with a fibroblast sheet (hEHs-Swiss) and monolayer hES cell (H9)-derived hepatocyte (hEHs-mono) on day 15. Scale bar = 25 μm .¹⁴⁶ Adapted from ref. 146 with permission from Elsevier, Copyright 2012.

for another few more days to form mature hepatocytes derived from hES cells (Figure 5.20(b)).¹⁴²

Hepatocytes derived from hES cells cultivated on 3D alginate matrices exhibited higher gene expression of hepatocyte-derived markers (*e.g.*, ApoF, TO, CYP7A1, and CYP3A) in comparison to the cells induced in 2D monolayer culture system.¹⁴²

CYP3A4 works the most critical task of the P450 enzymes in the human liver system. Then, CYP3A4 activity was investigated on HepG2 cells (positive control; a human hepatocyte cell line) and hepatocytes derived from hES cells, which were cultured on 3D alginate scaffolds to examine the potentially ameliorated hepatic function.¹⁴² Hepatocytes derived from hES cells that were cultivated on 3D alginate matrices exhibited extensively higher activity of CYP3A4 in comparison to hepatocytes cultivated on 2D monolayer system at a low inoculating density (0.5×10^6 cells per well).¹⁴² These findings indicate that the 3D cultivation of hepatocytes derived from hES cells on

alginate matrices ameliorates hepatocyte maturation and facilitates hepatic gene expression and function.

It is difficult to induce differentiation into fully functional hepatocytes using a typical 2D cultivation system. Dynamic 3D perfusion cultivation has the advantages of differentiation into the mature hepatocyte and enhancement of the hepatic function of primary adult hepatocytes.¹⁴⁷ Then, Miki and his colleagues investigated the possibility of hollow fiber-based 3D perfusion bioreactor system for induction of the hepatic differentiation of hES cells using a cocktail protocol of multistep growth factors and the Type E induction protocol.¹⁴⁷ Hepatic gene expression (*e.g.*, ASGPR-1, CYP3A4, albumin, and alpha-1 antitrypsin) was extensively enhanced in hES cells induced in the hollow fiber-based 3D perfusion bioreactor system.¹⁴⁷ Ammonia metabolism and albumin production were further observed in the hollow fiber-based 3D perfusion bioreactor system. The gene expression of cytochrome was found to increase in response to rifampicin.¹⁴⁷ Immunohistochemical assay indicated the structural formation of biliary marker-positive and hepatic cells on the hollow fiber bioreactor system.¹⁴⁷ Therefore, the 3D perfusion bioreactor promote the functional maturation of hepatocytes derived from hES cells.

Tissue engineering of liver needs the clustering of many kinds of liver cells within their niche microenvironments in a spatially designed structure, which enables vascularization of the scaffold *in vivo* where viability and hepatic function are maintained. Du and his colleagues investigated the entrapment of cells within separate sites in multi-component hydrogel fiber system as well as protocols for assembling fibers to generate 3D-patterned tissue scaffolds using the Type E induction protocol.¹⁴⁸ hiPS cells were induced into endothelial cells and hepatocytes. The endothelial cells and hepatocytes, which were derived from hiPS cells were entrapped within the assembled fibers, which were spun from the surface between two different charged-polyelectrolytes (alginate and chitin), thereby forming endothelialized liver tissue scaffolds (Figure 5.25A).^{148,156} In this protocol, the endothelial cells and hepatocytes were suspended in water-soluble COL (WSC-col) and chitin containing galactose (WSC-Gal), respectively. The droplet of WSC-col (polycation) including endothelial cells and WSC-gal (polycation) including hepatocytes, alginate (polyanion) solution, were located side-by-side (Figure 5.25A).^{148,156} Each polyelectrolyte droplet was enabled to be in contact with the adjacent droplets, forming four interfaces, which were fused to generate nascent polyelectrolyte fibers with four domains. Several polyelectrolyte fibers were spun simultaneously, merged, and spooled to form hydrogels where hepatocytes and endothelial cells, which were derived from hiPS cells were encapsulated (Figure 5.25A).¹⁴⁸ The existence of endothelial cells in the assembled fiber scaffolds extensively enhanced hepatocyte functions *in vitro* and promoted vascularization of the scaffolds when transplanted in mouse partial hepatectomy models (Figure 5.25B).¹⁴⁸ The *in vivo* investigations indicated that the integration of the scaffolds with the host vasculature generated, as found by the existence

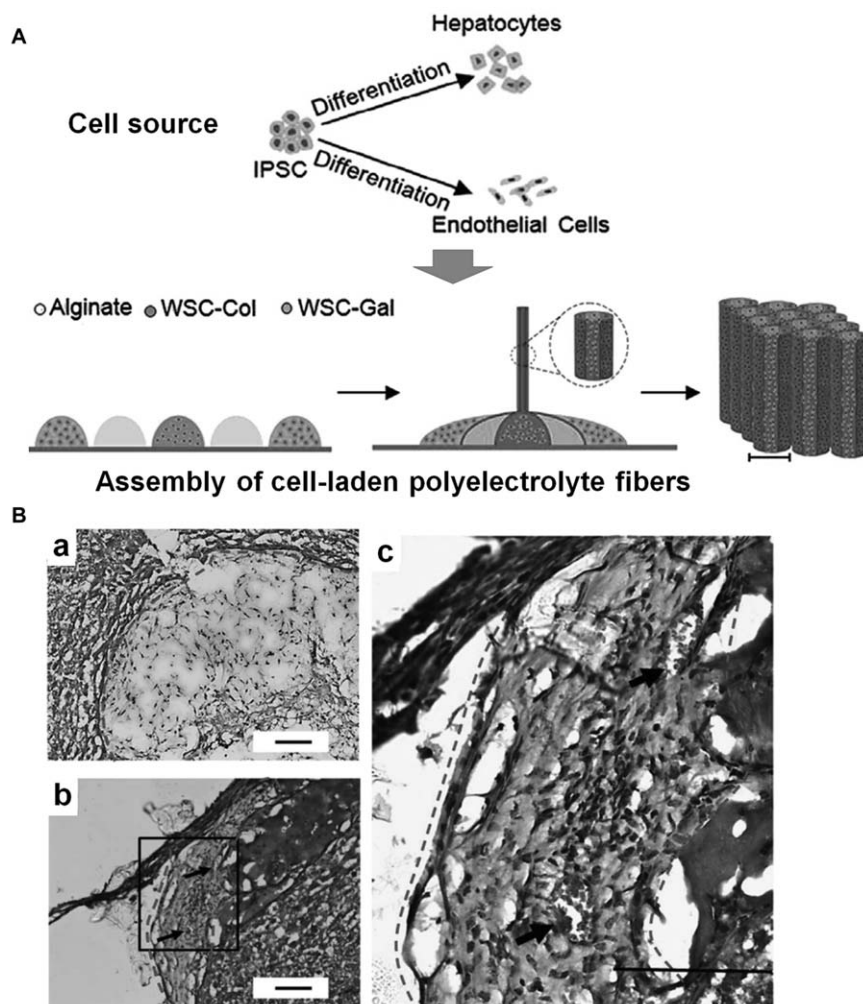


Figure 5.25 Co-cultivation of endothelial cells and hepatocytes derived hiPS cells in multi-component hydrogel fibers. (A) hiPS cells were induced into hepatocytes and endothelial cells. The differentiated cells were mixed either with chitin and COL (WSC-COL) for endothelial cells or with chitin and galactose (WSC-Gal) for hepatocytes, and droplets were located in an array with droplets of alginate solution. Endothelial cells or hepatocytes derived from hiPS cells were entrapped in the multi-interfacial polyelectrolyte complex (MIPC) fibers, which were spun from the interfaces between chitin and alginate. The fiber was fused to generate multi-component fibers. (B) Histological assay of the cryosections prepared from the explants of hepatocytes co-cultivated with endothelial cells and of hepatocytes alone. The dotted line marks the boundary of the fibrous scaffold transplanted in the partially hepatectomized host liver. The section was stained with H&E. (a) Section of the hepatocyte alone group. (b) Section of hepatocyte co-cultivated with endothelial cell. (c) Higher magnification of the boxed region in (b). Arrows in (b) and (c) show blood vessels. Scale bar = 100 μm .¹⁴⁸ Adapted from ref. 148 with permission from Elsevier, Copyright 2011.

of human albumin in mouse serum. The existence of endothelial cells in the scaffolds extremely enhanced hepatic functions *in vitro* and facilitated vascularization of scaffolds including hepatocytes derived from hiPS cells, which were transplanted in mouse partial hepatectomy models.

Hepatocytes derived from hPS cells prepared on appropriate materials should be attractive cell source for hepatitis research, toxicology, and drug screening as well as for future advances toward the translational medicine using hepatocytes derived from hPS cells. Recently liver bud-like tissue has been developed by several researchers,^{157,158} which represents 3D vascularized artificial liver. The author hopes biomaterials will also involve to develop sophisticated liver bud-like tissue using hPSCs.

5.4.3 Effect of Cell Culture Biomaterials on hPS Cell Differentiation into Hepatocytes

Lee and his colleagues prepared hybrid hydrogels (semi-interpenetrating network [IPN]) of polyethylene glycol [PEG]/HyA of varying elasticity (0.2–25 kPa) and compared them with a native liver (2–6 kPa), to establish more mature liver models, which preserve liver functions *in vitro*.¹⁵⁹ HepaRG cells (hepatocyte progenitor cells), either alone or with supporting cells (human sinusoidal endothelial cells and fibroblasts), were encapsulated in the biodegradable semi-IPN PEG/HyA hydrogels. The elastic modulus of the 3D liver dynamically changed during cultivation because of the combined effects of prolonged degradation of hydrogels and ECM formation provided by the supporting cells.¹⁵⁹ As a result, when the elastic modulus of the 3D liver model converges similar to the elastic modulus of the *in vivo* liver (2–6 kPa), both functional and phenotypic maturation of the 3D liver were achieved, while drug metabolism, cytochrome p450-3A4 activity, albumin secretion, and hepatic gene expression were increased.¹⁵⁹ The 3D liver model was expanded to applications with hES-derived hepatocytes (Hepatocyte-S, Nexel) and primary human hepatocytes (HFC476), and it supported prolonged hepatocyte function and survival in long-term cultivation.¹⁵⁹ The present 3D hydrogel model may represent extensive progress in preparation of a biomimetic liver system to mimic liver tissue remodeling, and supports a versatile platform in drug development and disease modeling.

It is interesting to investigate which ECM materials are suitable for differentiation of hPS cells into hepatocytes, because ECM provides critical cues for cell induction, migration, and expansion. Kanninen and his colleagues evaluate ECM molecules secreted by human liver progenitors (HepaRG cells).¹⁶⁰ Production of FN, LN α 2, LN α 5, LN β 1, LN β 2, LN γ 1, COL type IV α 1, COL type IV α 2, and COL type IV α 5 was observed from RT-PCR and immunofluorescence assay (Figure 5.26).¹⁶⁰ Therefore, they selected FN, LN-521 (composed of LN α 5, β 2, and γ 1; LN-11), and LN-511 (composed of LN α 5, β 1, and γ 1; LN-10) as coating materials of culture matrices for DE cells derived from hPS cells. The LN-521 and LN-511 either alone or in combination were found to support the hepatic specification, whereas FN

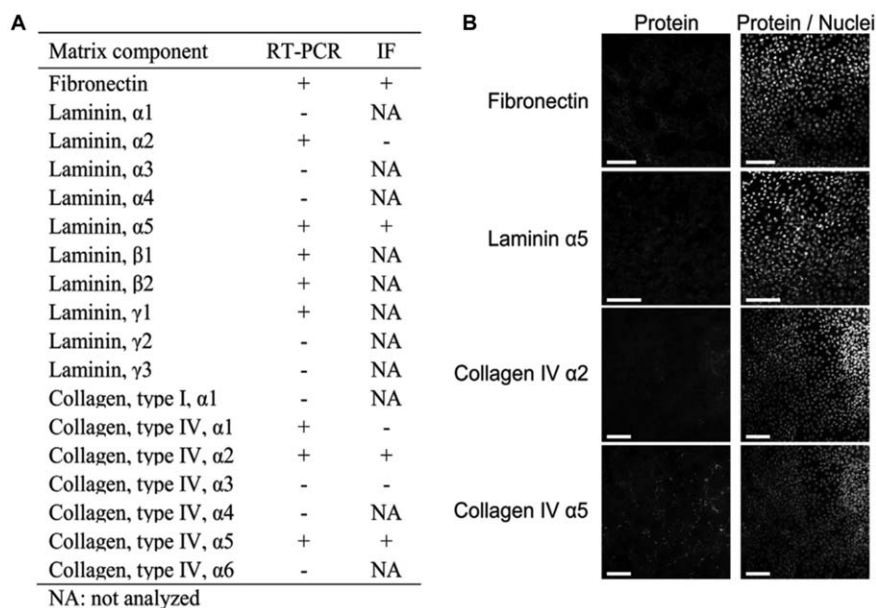


Figure 5.26 Characterization of the ECM components produced by HepaRG (human liver progenitor) cells. (A) ECM components secreted by HepaRG cells analyzed using RT-PCR and immunofluorescence (IF) evaluation. (B) Expression of FN, LN α 5, COL type IV α 2, and COL type IV α 5 on the HepaRG cells analyzed by immunofluorescence method. Scale bars = 100 μ m.¹⁶⁰
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was not a vital matrix protein for the DE cells derived from hPS cells. The expression of the LN-521/511-specific integrins (α 3 β 1 and α 7 $\times$ 1 β 1) was found to enhance during the DE induction and hepatic specification.¹⁶⁰ The hepatic cells induced differentiation on LN-coating surface exhibited the enhancement of liver-specific markers both protein levels and mRNA levels. The haptic cells on LN-coating surface showed cytochrome P450 enzyme activity, stored glycogen, and secreted human albumin.¹⁶⁰ It is concluded that LN-521 and LN-511 are preferable stage-specific matrices to induce the hepatic specification of DE cells derived from hPS cells.

Decellularized liver ECM has been also used for stem cell differentiation into hepatocytes. It was reported that hBMS cells cultured on decellularized liver ECM increased hepatic gene expression on hepatocyte-like cells differentiated from hMS cells.¹⁶¹ The hepatocytes differentiated from mouse BMS cells by coculturing with decellularized liver scaffolds showed enhanced hepatic marker expression compared to hepatocyte-like cells generated in conventional 2D culture system.¹⁶² Furthermore, the bioengineered liver prepared from decellularized liver scaffold and iPS cells, hepatocytes derived from iPS cells (Heps-iPS cells), has been investigated as an alternative method to cure liver disease. However, there was no report on both the interaction of

Heps-iPS cells with a liver ECM and the assay of recellularized Heps-iPS cells into the liver scaffold. Therefore, Park and his colleagues prepared porcine Heps-iPS cells, which expressed several hepatic markers such as albumin and α -fetoprotein, and exhibited hepatic functionalities, including ICG metabolism, LDL uptake, lipid accumulation, and glycogen storage.¹⁶³ Supplementation of ECM from porcine decellularized liver including liver-derived growth factors activated the albumin expression of porcine Heps-iPS cells during induction procedures. The Heps-iPS cells were reseeded into decellularized liver scaffold, and the recellularized liver was cultivated using a continuous perfusion system. The recellularized liver scaffold was implanted into rats for a short term, and the grafts showed hepatocyte markers and did not rupture.¹⁶³ These findings provide some examples on generation of bioengineered liver using stem cell and decellularized scaffold.

5.5 Differentiation into Insulin-secreting β Cells

It is a challenge to prepare insulin-secreting and glucose-responsive β cells from hPS cells *in vitro*,^{164–167} because typical β -like cells developed in the literature are not so mature, although DE and pancreatic progenitors can be induced from hPS cells with high purity.^{14,168–171} Three to four months are necessary for the cells to induce into mature and functional β cells when the cells are implanted into a rodent animal model.^{168,169} At present, the mechanism of induction into β cells *in vivo* is not clearly known, and it is not clear whether this mechanism of *in vivo* induction happens in human tissues. Previously generated cells had abilities to produce insulin. However, the secreting insulin amount was extremely less than that produced by primary human β cells in islets, and the cells could respond to no glucose levels.^{14,171–176} It is known that only mature β cells are limited to be able to produce insulin in response to glucose concentration quickly and subsequently shut off insulin secretion to avoid hypoglycemia. In the recent past, two valuable methods have been reported to prepare mature β cells from hES cells, which produce insulin by responding to glucose amount in local niche, which is similar characteristic to primary human β cells in islets. Rezanian and his colleagues¹⁵ published one of these methods, an excellent method for inducing hES cells into mature β cells using a seven-stage method (Figure 5.27(a)) with the Type E induction protocol.¹⁵

hES cells (H1) was inoculated on Matrigel-coated plates and cultivated in induction media including several growth factors and small molecules, which depended on the stage of induction, to prepare functional β cells. GSK3 β inhibitor, MCX-928, and GDF8 were used to induce hES cells into DE with 98–99% CXCR4⁺/FOXA2⁺ expression (DE, stage 1 in Figure 5.27(a)). Stage 1 cells were subsequently cultivated in media including BMP receptor inhibitor, FGF-7, and ascorbic acid (vitamin C) for 48 h for induction into the cells of the primitive gut tube (PGT, stage 2 in Figure 5.27(a)) that showed PDX1 expression. Then, stage 2 cells were cultivated in media including ascorbic acid, TPB (BMP receptor inhibitor), ITS-X, LDN193189, retinoic

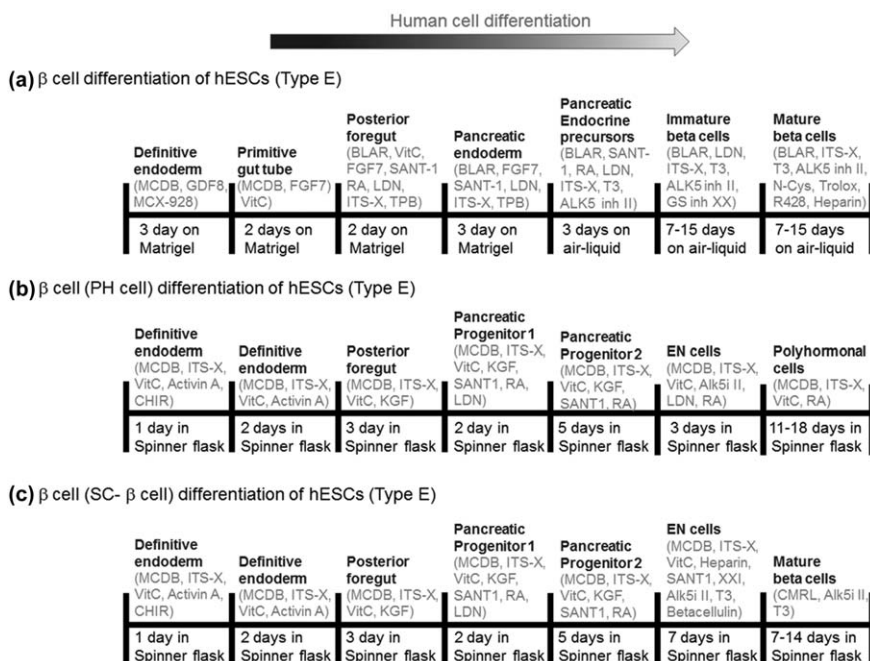


Figure 5.27 Timelines of protocols for differentiation of hPS cells into insulin-secreting β cells. (a) Induction methods reported by Rezania and his colleagues.^{5,15} (b and c) Differentiation protocols developed by Pagliuca and his colleagues.^{5,14} Adapted from ref. 5 with permission from Elsevier, Copyright 2016.

acid, SANT-1, and FGF-7 for 48 h for induction into posterior foregut cells (PF, stage 3 in Figure 5.27(a)).¹⁵ Stage 3 cells were further cultivated in media including TPB, ITS-X, LDN193189, retinoic acid, SANT-1, FGF-7, and ascorbic acid at some specific concentrations (PE, stage 4 in Figure 5.27(a)) for 72 h for induction into NKX6.1⁺/PDX1⁺ pancreatic endoderm cells. The addition of ascorbic acid at early stages of induction extensively generated NKX6.1⁺/PDX1⁺ pancreatic progenitors expressing low NGN3 marker and its downstream targeted markers (stage 4 in Figure 5.27(a)).¹⁵

Then, the stage 4 cells were processed with Y-27632 to make single cells. The cells were cultivated on membrane filter inserts having pore size 0.4 μ m with 5–10 μ L per spot for 0.25–0.5 $\times 10^6$ cells per spot at an air–liquid interface. Each spotted area was made to be 0.1–0.2 cm in diameter, and around 10–15 spots were located in each well of a six-well insert. Stage 4 cells were subsequently cultured in media including heparin, zinc sulfate, ALK5 inhibitor II, T3 (thyroid hormone, 3,3',5-triiodo-L-thyronine sodium salt), ITS-X, LDN193189, retinoic acid, and SANT-1 for 72 h for induction into NEUROD1⁺/NKX6.1⁺/PDX1⁺ pancreatic endocrine precursor cells (Figure 5.27(a)).¹⁵ In this method, the induced hES cells were cultivated at the air–liquid interface beginning at stage 4. However, the induction efficiency of

cells cultivated on conventional culture dishes or at the air–liquid interface did not appear to differ from their experimental results. Then, it seems not to be important to select air–liquid interface cultivation in the method.

Pancreatic progenitors were subsequently induced using a combination of some molecules, such as thyroid hormone (T3), BMP receptor inhibitor (LDN, TPB, and FGF-7), and ALK5 (TGF β receptor) inhibitor (ALK5 inh II), which led to upregulated expression of *NGN3* and a larger cell population co-expressing *NKX2.2*, *NEUROD1*, *NKX6.1*, and *PDX1* (PEP, stage 5 in Figure 5.27(a)).¹⁵ Continuous treatment with T3, BMP receptor inhibitor, and ALK5 inhibitor and with the addition of a notch inhibitor (gamma secretase inhibitor XX, GSiXX) made the creation of *NEUROD1*⁺/*NKX6.1*⁺/*PDX1*⁺ cells expressing insulin but not somatostatin or glucagon (stage 6 in Figure 5.27(a)).¹⁵ Then, the cells were treated with R428, an AXL inhibitor as well as T3 and ALK5 inhibitor, which resulted to produce functional β cells (*NEUROD1*⁺/*NKX6.1*⁺/*PDX1*⁺/*MAFA*⁺/cells), which were insulin⁺/somatostatin[−]/glucagon[−] (stage 7 in Figure 5.27(a)).¹⁵ The functional β cells derived from hES cells exhibited the secretion of glucose-stimulated insulin, which resemble to that of β cells in human islets cultivated *in vitro*.

Dynamic analysis of glucose stimulation indicated differences and similarities between the primary human β cells and functional β cells derived from hES cells. Especially, the functional β cells derived from hES cells immediately treated diabetes in mice within 40 days that was almost four times better than pancreatic progenitors.¹⁵ At this moment, the functional β cells derived from hES cells are not completely the same to mature human β cells. On the other hand, the ability of the functional β cells obtained from hES cells for *in vivo* glucose-responsive insulin secretion indicates high ability as a source of cadaveric islets or pancreatic progenitor cells for the cell therapy of diabetes. It should be valuable to investigate hES cells cultivated on materials immobilized with appropriate nanosegments or coated with appropriate ECMs and induced into functional β cells in the cultivation media used in the β cell induction method.

Pagliuca and his colleagues created an another protocol to prepare monohormonal and glucose-responsive insulin-producing cells from hiPS cells and hES cells using a six-stage method; the resulting cells expressed several important β cell markers and exhibited β cell ultrastructure after the sequential modulation of several signaling pathways in suspension cell cultivation in spinner bioreactors.¹⁴ Cell clusters (100–200 μ m in diameter) from hiPS cells (hiPSC-2 and hiPSC-1) and hES cells (HUES8) were differentiated into DE, and 94–96% of the cells showed Sox17⁺ (stage 1 in Figure 5.27(b)). Early pancreatic progenitors (PP1) were generated by induction of the DE cells in media including FGF-7 (KGF) for 72 h and subsequently in media including PdbU (phorbol 12,13-dibutyrate, a protein kinase C activator), LDN193189 (SHH pathway antagonist), SANT1 (hedgehog inhibitor), retinoic acid, and KGF for 48 h; this protocol resembles the protocol reported by Rezania and his colleagues.¹⁵ Late pancreatic progenitors (PP2) were produced by culture of early pancreatic progenitors in

media including SANT1, retinoic acid, and KGF for 5 days, and the resulting cells showed expression of NKX6.1 and PDX1 (PP2 in Figure 5.27(b)).¹⁴ These pancreatic progenitors were successively able to produce functional β cells 3–4 months after implantation into mice.^{14,168,169} Some investigators found that these pancreatic progenitors (PP2) could be differentiated into insulin-secreting (INS^+) cells, such as SST (somatostatin)⁺/ INS^+ polyhormonal (PH) cells or GCG (glucagon)⁺/ INS^+ cells (Figure 5.27(b)).^{14,169,171–173,177,178} PH cells are similar to human fetal β cells but not similar to mature β cells.^{14,172} Then, PH cells could not exhibit insulin secretion by glucose stimulation.

Then, a novel method was reported to make functional C-peptide⁺/ NKX6.1^+ β cell using pancreatic progenitors.¹⁴ At first, the pancreatic progenitor (PP1) was cultivated in media including SANT1 and KGF with a low concentration of retinoic acid for 5 days to get a large percentage (>50%) of $\text{PDX1}^+/\text{NKX6.1}^+$ pancreatic progenitor (PP2) (Figure 5.27(c)). Pagliuca and his colleagues cultivated the pancreatic progenitors (PP2) in media including Alk5i, betacellulin, heparin, XXI, T3, SANT1, and retinoic acid for 7 days to make C-peptide⁺/ NKX6.1^+ cells (EN cells in Figure 5.27(c)), after complete analysis of the exposure time and concentration of small molecule antagonists and agonists as well as growth factors. Then, these cells were cultivated in media including CMRL, Alk5i, and T3 supplement to prepare functional β cells.¹⁴ The function of these hPS cell-derived β cells mimicked that of human islets both *in vivo* and *in vitro* and, shortly after implantation, secreted human insulin into the blood of mice in a glucose-regulated way for at least 4 months. The implantation of the functional hPS cell-derived β cells improved hyperglycemia in diabetic mice (Figure 5.28), suggesting the possible use of the cells for *in vivo* implantation therapy for diabetes.¹⁴

Encapsulation of donor islets into hydrogels is one of the strategies for islet implantation that deletes donor islets from the host immune response reaction. The replacement of donor islets by hES cell-derived islets would also need the strategy of immune-isolating hPS cells by entrapment into hydrogels. An extensive consideration should be done for the effect of surrounding biophysical environment on the pancreatic differentiation of hPS cells, especially for the effect of capsule biophysical properties of hydrogels. Therefore, Richardson and his colleagues evaluated the effect of capsule characteristics (barium alginate hydrogel) on viability, growth, and differentiation of entrapped hES cells during pancreatic differentiation.¹⁷⁹ They selected to use barium alginate hydrogels instead of calcium alginate, which has been widely used in hPS cell entrapment (Figure 5.29).¹⁷⁹ This is because barium binds to alginate with higher affinity than calcium, which results in more robust encapsulation of the cells. Calcium ion can be easily replaced by monovalent cation such as sodium, resulting in weakening the calcium alginate hydrogels over time. The barium alginate encapsulation has been widely used for islet entrapment into the hydrogels for type 1 diabetes treatment.^{180,181} Their results showed that biomaterial characteristics could significantly regulate pancreatic differentiation, especially careful tuning of encapsulating properties even in the presence of soluble chemical cues for

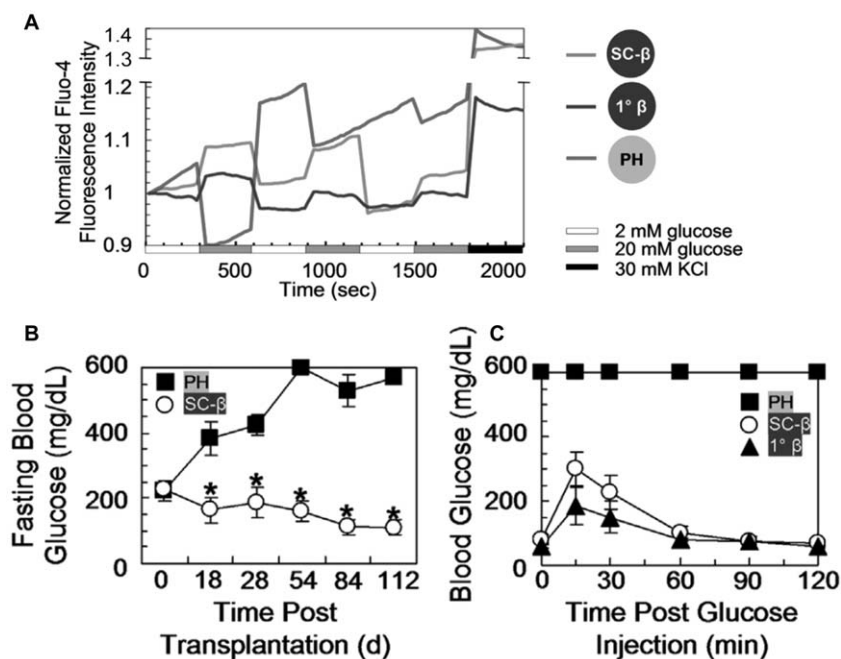


Figure 5.28 Treatment of hyperglycemia in diabetic mice by implantation of β (SC- β) cells derived from hES cells. (A) Dynamic normalized Fluo-4 fluorescence intensity (cytosolic Ca^{2+} concentration intensity) in SC- β cells, primary β cells ($1^\circ\beta$), and polyhormonal (PH) cells injected sequentially with 2, 20, 2, 20, 2, and 20 mM glucose and 30 mM KCl *in vitro*. (B) Fasting blood glucose evaluation in progressively diabetic NRG-Akita mice implanted with SC- β cells (5×10^6 cells; open circles) or PH cells (5×10^6 cells; closed squares). Glucose concentrations were saturated at 600 mg dl^{-1} . (C) Blood glucose assay in mice implanted 137 days prior with SC- β cells (open circles) or PH cells (closed squares) and in an independent cohort of mice implanted 34 days prior with human islets ($1^\circ\beta$, 4000 IEQ; closed triangles).¹⁴ Adapted from ref. 14 with permission from Elsevier, Copyright 2011.

pancreatic induction. Capsules in the range of 4.0–7.0 kPa of barium alginate maintained cell viability and growth, while encapsulation of higher stiffness restricted cell expansion.¹⁷⁹ The enhancement of stiffness extensively suppressed induction into pancreatic progenitors, while an increase in capsule stiffness facilitated induction at the intermediate DE stage. An enhancement in pSMAD/pAKT levels with biomaterial stiffness is the main cause of increase of DE differentiation, which is evaluated from signaling pathway analysis.¹⁷⁹ Furthermore, SHH inhibition promote more efficient differentiation under softer hydrogel condition, which is important for successful pancreatic progenitor differentiation. In other words, the enhancement of alginate capsule stiffness promotes TGF- β signaling during the DE stage, which enhances induction into DE.¹⁷⁹ However, increased alginate hydrogel

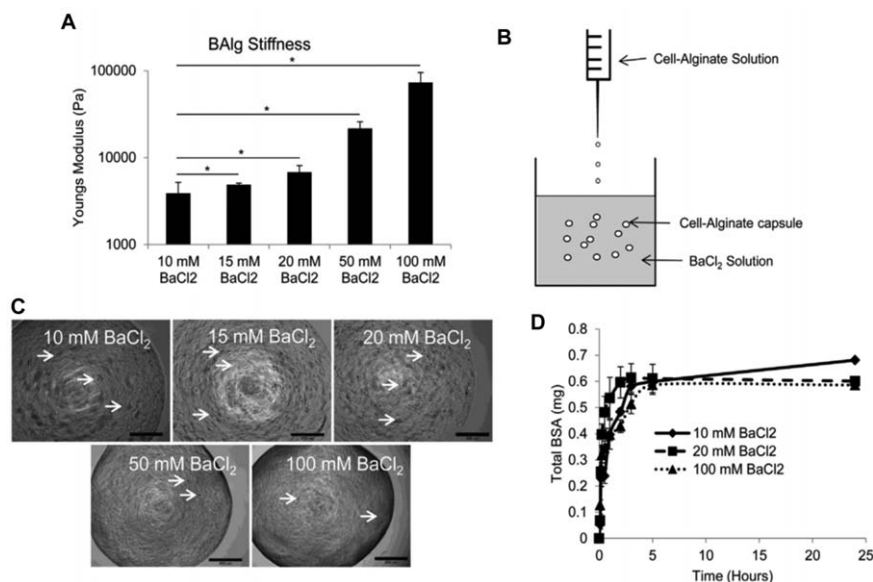


Figure 5.29 Characterization of barium alginate capsules. (A) Elastic moduli (Pa) of alginate hydrogels crosslinked with 10–100 mM BaCl₂ analyzed by atomic force microscopy nano-indentation. (B) Barium alginate encapsulation method of hES cells with the desired BaCl₂ concentration. (C) Micrographic pictures of hES cells entrapped into barium alginate hydrogels using 10–100 mM BaCl₂ after proliferation of hES cells for 6 days to generate colony formation. The arrows show colonies. Scale bar is 450 μm. (D) BSA released from barium alginate hydrogel capsules over 24 h.¹⁷⁹

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stiffness also facilitated SHH signaling at the pancreatic progenitor stage that reduced the differentiation into pancreatic progenitors.¹⁷⁹ Overall, cell growth and pancreatic induction of hES cells in the barium alginate hydrogels was found to be optimal in the stiffness range around 4.0–7.0 kPa.

It should be valuable to investigate hPS cells cultivated on materials immobilized with appropriate nanosegments or coated with optimal ECMs and induced into functional β cells in the cultivation media used in the above-described method. At this moment, it is difficult to conclude whether the protocols reported by Rezania *et al.*¹⁵ or by Pagliuca *et al.*¹⁴ produce cells, which are more resemble human primary β cells. It is important for the same investigator to prepare β cells using both protocols and analyze cell function and proceed *in vivo* transplantation assay.

5.6 Conclusions and Perspectives

Stem cell investigation has targeted on development of stem cells, which can be induced into desired lineages of cells. The specific materials should

contribute to the pluripotency of hES and hiPS cells and further regulate the differentiation fate of hES and hiPS cells.

At present, some sophisticated protocols have been reported for induction into appropriate lineages of cells, including cardiomyocytes,^{17,18} dopamine-secreting TH⁺ cells,¹⁶ and insulin-secreting β cells.^{14,15} However, it is not known which cell cultivation materials are adequate for inducing differentiation into the specific cell lineages where the biomaterials are being created by different groups of investigators.

Some excellent materials have been reported for the cultivation and differentiation of hPS cells in 2D cultivation conditions. On the other hand, it is not easy to prepare a large amount of hPS cell-derived cells in a desired lineage in 2D cultivation conditions. It is important to design microcarrier grafting or coating biomaterials or to prepare microcarrier biomaterials for the optimal and efficient induction of hPS cells on the microcarriers.

The present hPS cell cultivation procedures utilize a batch-type process that uses disposable cell cultivation microcarriers or dishes. Subsequently, this process is laborious and expensive. Currently, a continuous cell cultivation condition for the proliferation of hPS cells was developed on thermo-responsive nanobrush interfaces.¹⁸² This system provides the microcarriers or plates immobilized on thermoresponsive nanosegments for the induction of hPS cells into desired cell lineages; the cells are able to be continuously harvested in the cultivation media by decreasing the temperature of the cultivation media that would extensively reduce the cost of hPS cell cultivation and induction.

At this moment, the clinical application of hPS cells is extensively restricted.¹⁸³ On the other hand, ocular diseases are the first human trials of hPS cells and Phase I/II trials exhibited promising safety results with some possible efficacy.^{184,185} The current clinical trials for hPS cell-based therapies extensively focusing on cure of retinal degeneration in the eye. The extremely pure GMP-graded induced cells from hPS cells that are prepared on optimal materials, would contribute to the ocular disease cure and other disease treatment.

The preparation of materials for hPS cell induction under xeno-free systems would be one of the most important outcomes in biomedical investigation for the positive shift from hPS cell study to translational medicine. The combination of cell cultivation materials and optimal induction methods for the induction of hPS cells into desired cell lineages would produce a high quantity of extremely pure GMP grade induced cells for use in tissue engineering and regenerative medicine.

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CHAPTER 6

Clinical Trials of Stem Cell Therapies Using Biomaterials

6.1 Introduction

Human pluripotent stem (hPS) cells, human induced pluripotent stem (hiPS) cells, and human embryonic stem (hES) cells appear to be valuable tools in the cure of injured organs or tissues because of their potential to induce differentiation into many cells derived from the three embryonic germ layers in the human body.

At present, clinical trials of stem cell therapy using hPS cells have only been reported for four cases according to the ClinicalTrials.gov database. These cases are (1) macular degeneration (namely Stargardt macular dystrophy and age-related macular degeneration), (2) acute myocardial infarction (AMI), (3) diabetes, and (4) spinal cord injury (Figure 6.1). Recently, hPS cell-based therapy in clinical trials has been studied.^{1–5} There are a few review articles that report the current situation of therapy using hES cells and hiPS cells.^{1–5} However, the articles do not consider the bioengineering points of view for these therapies, such as hPS cell cultivation and induction protocols, biomaterials for hPS cell differentiation, transplantation method including biomaterial usage, and conditions of hES cells and hiPS cells. In particular, implantation conditions such as cell suspension injection or cell monolayer implantation without and with materials (hydrogels or scaffolds) at injected sites have not been discussed. Then, we discuss the current situation of stem cell therapy using hPS cells for patients with (a) myocardial infarction (MI) (Section 6.2) and (b) macular degeneration (Section 6.3), considering the bioengineering points of the therapy. Moreover, we consider clinical trials using adult or human fetal stem cells such as human mesenchymal stem (hMS) cells that are prepared to cure patients with these diseases.

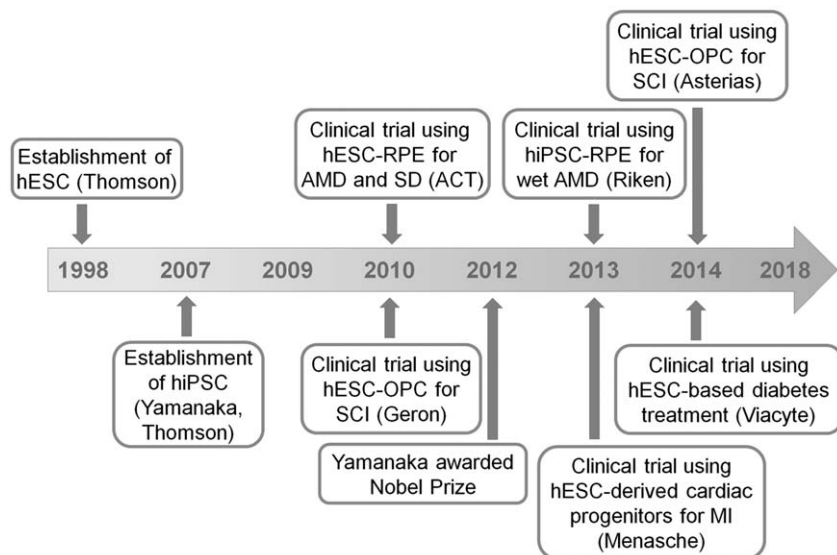


Figure 6.1 History of hPS cells and clinical trial using hPS cells.

The goal of this chapter was (1) to describe the use of materials in clinical and preclinical works of hPS cells, hMS cells, and human fetal stem cells, (2) to describe the gap between clinical trials and fundamental research using human stem cells, (3) to describe the current situation of clinical trials of hPS cell-based therapy in comparison to clinical trials using hMS cells and human fetal stem cells, and (4) to consider the bioengineering trends to facilitate hPS cell, hMS cell, and human fetal stem cell therapy in translational medicine.

6.2 Stem Cell Therapy for Myocardial Infarction (MI) in Clinical Trials

Cardiovascular disease is regarded as the top cause of disability and death in the USA, claiming more lives each year than HIV, diabetes mellitus, cancer, and accidents combined.⁶ Ischemic heart disease is the top contributor to cardiovascular mortality or morbidity; there are more than 1 million MIs each year in the USA, whereas 5 000 000 patients suffer from chronic heart failure.⁷ Although death rates are reported to have improved enormously over the last four decades, novel treatments are much needed for patients who go on to develop ventricular dysfunction.^{6,8} Over the last decade, stem cell implantation has emerged as an attractive therapeutic method for chronic or acute ischemic cardiomyopathy. In this chapter, we discuss the methodologies and results of clinical trials of therapy for patients with acute MI (AMI) using hPS cells and hMS cells as well as human fetal stem cells (Figure 6.2).⁹

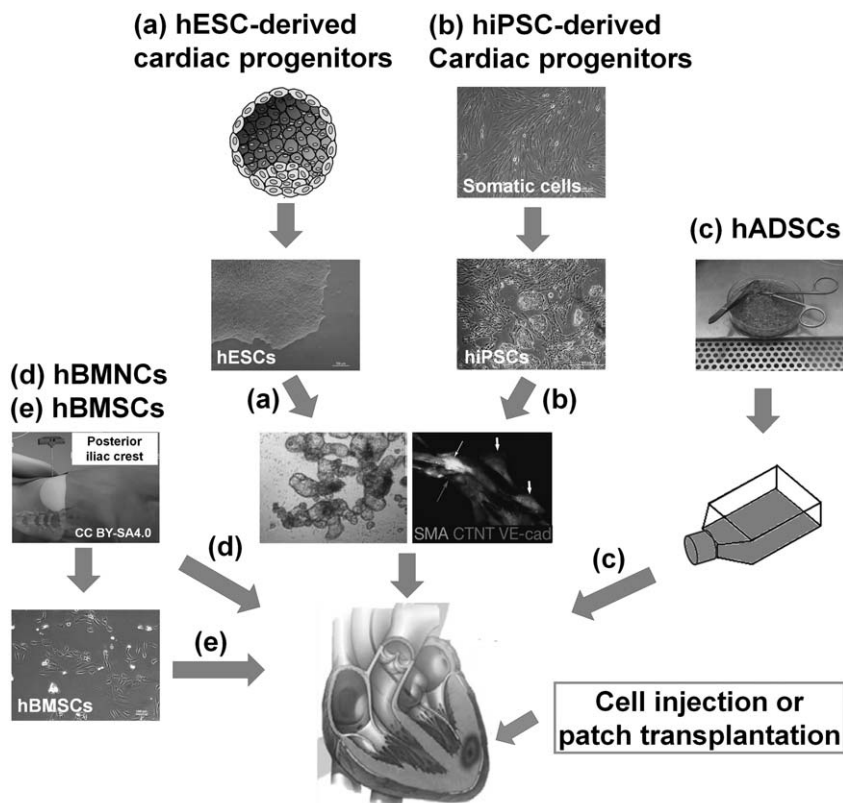


Figure 6.2 Illustrated picture of stem cell therapies for myocardial infarction diseases. (a) hES cells were obtained from inner cell mass of embryo and induced into cardiac progenitors. Cardiac progenitors were intracoronary infused or the patch including hES cell-derived cardiac progenitors was made and implanted into ischemic heart. (b) hiPS cells were generated from transfection or transduction of pluripotent proteins or genes into somatic cells, fetal stem cells, or adult stem cells and induced into cardiac progenitors. Cardiac progenitors were intracoronary infused or the patch including hiPS cell-derived cardiac progenitors was made and implanted into ischemic heart. (c) hADS cells were purified from fat tissue and subsequently intracoronary infused. (d) hBMN cells were sorted from bone marrow and subsequently intracoronary infused where bone marrow was isolated from posterior iliac crest. (e) hBMS cells were isolated by culture of hBMN cells on TCP plates and subsequently intracoronary infused.⁹ Adapted from ref. 9 with permission from Springer, Copyright 2017.

6.2.1 Clinical Therapies for MI Using hES cells

6.2.1.1 Fibrin Patch Including hES Cell-derived Cardiac Progenitors

hPS cells are attractive cell source for stem cell therapy and drug discovery because of their high potential to induce differentiation into variety of cell

types in our bodies.^{10–12} hES cell-derived cardiac progenitors were used for the recovery of the function of infarcted heart (NCT02057900). In this trial, the cells differentiated from hES cells were not directly implanted into injured site; while the cells were directly implanted into subretinal sites for a patient with macular degeneration,^{13–15} a fibrin patch including hES cell-derived cardiac progenitors was implanted into the AMI patients (Figure 6.3).^{16,17}

The fibrin patches were prepared by Menasche and his colleagues as follows (Figure 6.3):^{16,17} (a) Sixteen lines of hES cells were expanded under good manufacturing practice (GMP) conditions. hES cells were cultured on murine fibroblasts at early stages of development and on clinical-grade fibroblasts during differentiation of hES cells into cardiac progenitors. Fetal calf serum and porcine trypsin were also used throughout the expansion. Sufficient amounts of hES cells were expanded and cryopreserved. Xeno-free culture medium and culture substrates such as recombinant vitronectin-coated dishes or laminin 521-coated dishes may improve their protocols.^{10,11} (b) hES cells were induced to the differentiation into cardiac progenitors by culture in medium supplemented with insulin-free B27 supplement, bone morphogenetic protein (BMP)-2, and SU-5402 (a fibroblast growth factor [FGF] receptor-specific tyrosine kinase inhibitor) on clinical-grade fibroblast feeder layer for 4 days. 45–65% of the differentiated cells showed the expression of SSEA-1 (stage-specific embryonic antigen-1). (c) The cardiac progenitors were sorted using magnetic-activated cell sorting (MACS), which targeted SSEA-1-displaying cells. The 95–99% of cardiac progenitors showing

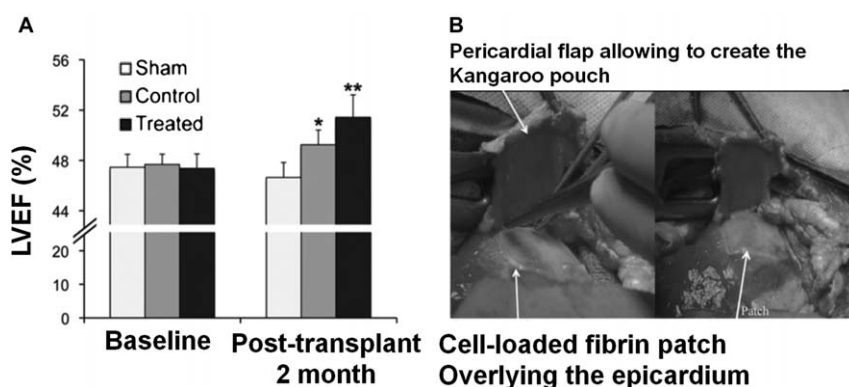


Figure 6.3 Implantation of a fibrin patch including hES cell-derived cardiac progenitors into rat (A) and human (B) of myocardial infarction. (A) LVEF shift between baseline and 60 days post transplantation. The LVEF of rats administered with the cell-loaded fibrin patch enhanced relative to LVEF of untreated rats.¹⁶ Copyright 2015. Adapted with permission from Oxford University Press. (B) Photo of implantation of the fibrin patch including hES cell-derived cardiac progenitors into the infarcted region of a patient heart, followed by covering the patch with a pericardial flap to make trophic factors into the infarcted heart.¹⁷ Adapted from ref. 17 with permission from Oxford University Press, Copyright 2015.

the surface marker of SSEA-1 were prepared after sorting by MACS. (d) The cardiac progenitors were investigated for safety, specially the loss of tumor-forming (formation of teratoma) potential. Teratoma formation was not generated when 100 times the highest clinical dose of cardiac progenitors was transplanted into immunodeficient mice.^{16,17} Cytogenic studies also proceeded by karyotype evaluation and examination by molecular cytogenetic techniques such as oligonucleotide based-array comparative genomic hybridization (array-CGH) and fluorescence *in situ* hybridization (FISH). They found no cytogenetic abnormalities in the cardiac progenitors in this study. (e) The fibrin patches including cardiac progenitor was generated in the following methods: ten million cardiac progenitor cells were injected into cell cultivation media (1.1 mL) including fibrinogen and inserted into agarose-coated plates (2.5 cm diameter). Four units of thrombin per 1.1 mL of cell cultivation media was added to the agarose-coated plates including fibrinogen and cardiac progenitors to form 25 μ L droplets. The solution in the agarose-coated plates was mildly agitated to bring about hydrogel polymerization. (f) The fibrin patches were applied to the epicardium, which was followed by covering the patches with a pericardial flap to hold trophic supports to the underlying cellular graft (Figure 6.3).

The efficacy of the fibrin patches including cardiac progenitor (1 cm² fibrin patch immobilized with 700 000 cardiac progenitors) was evaluated echocardiographically in a rat model of MI in a preclinical study. The LV (left ventricular) ejection fractions of the rats operated with the fibrin patches without or with cardiac progenitor cells and without operation (sham) are described in Figure 6.3.¹⁶ The LV ejection fraction improved after 8 weeks in the rats treated with the fibrin patches including cardiac progenitor cells, in comparison with an abnormal LV ejection fraction of less than 50% and a normal (reference) LV ejection fraction of about 55%.¹⁶

6.2.1.2 Clinical Trials With Fibrin Patch Including Cardiac Progenitors Derived from HES Cells

In human clinical trials, a 20 cm² area of autologous pericardium was taken, and the posterior half was sutured to the epicardium along the border of the infarct area of patients with severe ischemic LV dysfunction. A pocket between the pericardial flap and the epicardium was generated in this process.^{16,17} The fibrin patches including cardiac progenitors derived from hES cells was then inserted into the pocket (Figure 6.3B), and sutures were used to anchor the anterior half of the pericardial flap to the peri-infarct-affected epicardium that secured the 'sandwiched' cell-laden patches to the injured site. The immunosuppressive drug of cyclosporine as part of an immunosuppressive plan was used for 8 weeks. After implantation, the patient's serum was observed to be negative for Class II antigens, with low reactivity against non-donor-specific Class I antigens. Twelve weeks after the operation, the cardiac functional conditions of the patient were found to be greatly improved. The patient exhibited an enhanced LV ejection fraction

(36% from an initial 26%), and data of a 6-min walking test indicated an enhancement from 350 m to 467 m. The akinetic infarct zone where the fibrin patch including cardiac progenitor cells derived from hES cells was attached, was found to be moderately hypokinetic.^{16,17}

6.2.1.3 Mechanism of Enhanced Function by Fibrin Patches

The mechanism of enhanced function of infarcted heart is considered to be due to paracrine effects. The functional benefits of cardiac progenitor cells derived from ES cells are kept over time, despite rapid loss of the implanted cells.¹⁸ The cardiac progenitor cells secrete EV (extracellular vesicles) and some growth factors in the infarcted sites of hearts, where EVs include microparticles and exosomes, which orchestrate paracrine therapeutic effects. The cardiac progenitors showing the SSEA-1 surface marker and the cardiac genes *Mef2c* and *Isl-1* were selected to be used and the mature cardiomyocytes were not used in this clinical trial.^{16,17} The cell source (*i.e.*, mature cardiomyocyte or cardiac progenitor cell), which is more powerful in treating infarcted heart function would be a point of discussion. Only 16 hES cell lines having various HLA (human leukocyte antigen) types were prepared and immunosuppressive treatment was used in this research. It is important to prepare more hES cells with different HLA (major histocompatibility class [MHC] class I and class II) types to treat diverse patients with this therapy.

At this time, no other clinical trials using hES or hiPS cells are reported for patients with MI. However, several animal studies on hES cell-based therapies were performed and the improved cardiac function was reported using infarcted animal models.^{19–21}

6.2.2 Clinical Therapy for MI Using Fetal and Adult Stem Cells

6.2.2.1 MI Therapy Using Fetal and Adult Stem Cells

AMI is a typical cause of death and disability, although some medical treatment, such as PCI (percutaneous coronary intervention) and CABG (coronary artery bypass grafting) have been used to ameliorate these effects. Myocardial ischemia leads to the loss of contractile tissues that disrupt the mechanical potential of the LV of AMI patients.²² In this case, human adult stem (hAS) cell treatment, which includes (a) cardiomyocyte creation and angiogenesis by hAS cells, (b) growth factor secretion from hAS cells, and (c) inhibition of cardiomyocyte apoptosis, has emerged as an alternative therapy to regenerate injured myocardium. In general, autologous bone marrow mononuclear (BMN) cells are administered with intracoronary implantation into an MI site 4–8 days after PCI procedures in AMI patients. Other cell sources, such as Wharton's jelly-derived MS cells, mononuclear cells from peripheral blood, cardiopoietic stem cells, and allogenic or autologous MS cells from bone marrow (BM), are also used for AMI therapy (Table 6.1). No clinical trial of human adipose-derived stem (hADS) cells for AMI therapy is reported in our database studies (Table 6.1).

Table 6.1 Clinical trials reported for stem cell implantation (intracoronary transfer) on patients with acute myocardial infarction.⁹ Adapted from ref. 9 with permission from Springer, Copyright 2017.^a

Clinical trial name	Cell type	Cell no. (BM volume)	Time for injection	Patient no. (control/cell transfer)	LVEF baseline (control/cell transfer)	LVEF increase (%) (control)	LVEF increase (%) (cell transfer)	Overall effectiveness	Clinical trial number	Ref. (year)
BOOST	Autologous BMNCs	25×10 ⁸ cells (128 mL BM)	4.8 days after PCI	30/30	51.3%/50.0%	0.7% after 6 months	6.7% after 6 months	LVEF increased in BMNC group	NCT00224536	24 (2004)
ASTAMI	Autologous BMNCs	(50 mL BM)	5–8 days	25/24	No data available	No data available	No data available	Not specified	NCT00199823	23 (2005)
—	Autologous BMNCs	68×10 ⁶ cells (50 mL BM)	6 days after AMI	50/50	41.9%/41.9%	7.6% after 6 months	7.6% after 6 months	No effect on BMNC transplantation	NCT00199823	46 (2006)
(REPAIR-AMI)	Autologous BMNCs	236×10 ⁶ (50 mL BM)	3–6 days after PCI	103/101	46.9%/48.3%	3.0% after 4 months	5.5% after 4 months	Contractile function improvement in BMNC group	NCT00279175	27 (2006)
REPAIR-AMI	Autologous BMNCs	Unknown	3–6 days after PCI	103/101	No data available	No data available	No data available	Effective	NCT00279175	28 (2006)
BOOST	Autologous BMNCs	25×10 ⁸ cells (128 mL BM)	4.8 days after PCI	22/20	51.3%/50.0%	3.1% after 18 months	5.9% after 18 months	No statistical difference after 18 months	NCT00224536	25 (2006)
STEMI	Autologous BMNCs	304×10 ⁶ cells (130 mL BM)	1 day after PCI	34/33	46.9%/49.1%	2.6% after 4 months	2.7% after 4 months	Significant reduction of MI size in BMNC group	NCT00264316	32 (2006)
REPAIR-AMI	Autologous BMNCs	(50 mL BM)	3–6 days after PCI	28/30	45.6%/47.8% by Doppler study	Not analyzed	NA after 4 months	Improvement in maximal vascular conductance capacity	NCT00279175	29 (2007)
BOOST	Autologous BMNCs	25×10 ⁸ cells (128 mL BM)	4.8 days after PCI	30/30	51.3%/50.0%	−3.2% after 61 months	−2.5% after 61 months	No improvement of LVEF by single dose BMNCs transplantation	NCT00224536	26 (2009)

REAGENT	Autologous BMNCs or CD34-CXCR cells	1.8×10^8 for BMNCs, 1.9×10^6 cells for CD34-CXCR cells	3–12 days after AMI	40/160		0% after 6 months	3% after 6 months	No significant difference between cell treated group and placebo group	NCT00316381	33 (2009)
REPAIR-AMI	Autologous BMNCs	50 mL BM	3–6 days after AMI	28/30	40.1%/38.8%	– 0.4% after 12 months	7.7% after 12 months	Effective if EF <48.9% on baseline	NCT00279175	30 (2009)
PROCYMAL	Allogeneic BMSCs	0.5, 1.6, and 5 million per kg	3–10 days after AMI	21/39	45.1%/47.3%	5.2% after 12 months	1.8% after 12 months	hMSC treatment, but not placebo, increased LVEF	NCT00114452	35 (2009)
REPAIR-AMI	Autologous BMNCs	(50 mL BM)	3–6 days after AMI	101/100	48.7%/45.4%	–1.8% after 2 years	4.7% after 2 years	Infarct size and regional contractility were improved in BMNC treated group	NCT00279175	31 (2010)
TAC-HFT	Autologous BMSCs or BMNCs	200×10^6 cells	Unknown	20/40	No data available	No data available	No data available	No data available	NCT00768066	34 (2011)
SCIPIO	CSCs	1×10^6	113 days after CABG	38/33	30.1%/30.3%	0.15% after 4 months	8.2% after 4 months	CSC treatment, but not control increased LVEF	NCT00474461	36 (2011)
LATE TIME	Autologous BMNCs	150×10^6 cells (80–90 mL BM)	2–3 weeks after AMI	26/55	45.3%/48.7%	3.5% after 6 months	0.5% after 6 months	No significant effect	NCT00684060	38 (2011)
HEBE	Autologous BMNCs or PBMNCs	300×10^6 (60 mL BM or 150–200 mL PB)	3–8 days after AMI	65/69 and 65/66	42.4%/38.6% for BMNCs, 42.4%/36.8% for PBMNCs	4% after 4 months	3.8% for BMMNC, 4.2% for PBMNC after 4 months	No effect	ISRCTN95796863	39 (2011)
BONAMI	Autologous BMMNCs	50 mL BM (100×10^6 BMMNCs)	7–10 days after AMI	49/52	37.0%/35.6%	4.3% after 3 months	3.3% after 3 months	Myocardial viability increased	NCT00200707	40 (2011)
CADUCEUS	Autologous CDCs	12.5 or 25 million cells (276 mg EBT)	1.5–3 months after AMI	8/17	41%/38.1%	1.1% after 6 months	2.0% after 6 months	Scar mass reduced	NCT00893360	41 (2012)

Table 6.1 (Continued)

Clinical trial name	Cell type	Cell no. (BM volume)	Time for injection	Patient no. (control/cell transfer)	LVEF baseline (control/cell transfer)	LVEF increase (%) (control)	LVEF increase (%) (cell transfer)	Overall effectiveness	Clinical trial number	Ref. (year)
TIME	Autologous BMNCs	150×10 ⁶ BMNCs	3 or 7 days after AMI	41/79	44.5%/45.2%	3.3% after 6 months	3.1% after 6 months	No effect	NCT00684021	37 (2012)
C-CURE	Autologous cardiopoietic stem cells from BMSCs	733×10 ⁶ cells	4–7 weeks after AMI	15/21	27.8%/27.5%	0.2% after	7% after	Feasible cardiopoietic stem cell therapy	NCT00810238	42 (2013)
SCAMI	Autologous BMNCs	324×10 ⁶ BMNCs	7 days after AMI	13/29	55.7%/53.5%	3.7% after 36 months by CMR	0.5% after 36 months by CMR	Improvement in LVEF in high-dose transplantation group	NCT00669227	43 (2013)
—	Autologous BM-derived MSCs	23 mL of BM	>25 days after AMI	36/33	52.3%/49.0%	1.6% after 6 months	5.9% after 6 months	Modest improvement in LVEF	NCT01392105	47 (2014)
CHINA-AMI	Autologous BMNCs	100×10 ⁶ BMNCs (80–100 mL BM)	6 days after AMI	14/22	57.1%/50.9%	–3.0% after 12 months	3.9% after 12 months	No significant effect of BMNCs transplantation	NCT01234181	44 (2015)
—	Allogenic WJ-MSCs	6×10 ⁶ WJMSCs (passage 3)	5–7 days after PCI	58/58	51.1%/52.0%	2.8% after 18 months	7.8% after 18 months	LVEF increased in WJMSCs group	NCT01291329	48 (2015)
REGENERATION-AMI	Autologous BMNCs	60×10 ⁶ BMNCs (100 mL BM)	0–1 day after PCI	45/55	48.9%/47.8%	2.8% after 1 year	5.1% after 1 year	Small non-significant improvement of LVEF in BMNCs group	NCT00765453	45 (2016)

^aBM, bone marrow; BMNCs, bone marrow-derived mononuclear cells; BMSCs, bone marrow-derived mesenchymal stem cells; CABG, coronary artery bypass grafting; CD34-CXCR, CD34⁺CXCR4⁺ bone marrow cells; CDCs, cardiosphere-derived cells; CSCs, c-kit-positive cardiac stem cells; EBT, endomyocardial biopsy tissue; PBLs, peripheral blood leucocytes; PCI, percutaneous coronary intervention; PNMCS, peripheral blood mononuclear cells; WJ-MSCs, Wharton's jelly-derived mesenchymal stem cells.

6.2.2.2 MI Therapy Using Human BMN Cells

Many clinical trials on human BMN (hBMN) cells and other fetal or adult cell sources for MI patients have been studied, including REGENERATION-AMI,²³ CHINA-AMI,²⁴ SCAMI,²⁵ C-CURE,²⁶ CADUCEUS,²⁷ BONAMI,²⁸ HEBE,²⁹ LATE TIME,³⁰ TIME,³¹ SCPIO,³² PROCHYMAL,³³ TAC-HFT,³⁴ REGENT,³⁵ STEMI,³⁶ REPAIR-AMI,^{37–41} BOOST,^{42–44} ASTAMI,⁴⁵ and other clinical trials^{46–48} (Table 6.1), after Orlic and his colleagues studied the first clinical trial of an intracoronary implantation of autologous hBMN cells for MI patients and reported enhancement of LV function.⁴⁹

A typical treatment is as follows: (1) recent MI patients with left ventricular dysfunction (typically with a left ventricular ejection fraction (LVEF) of <50%) who had performed successful PCI with stent placement are chosen, after approval by the relevant Institutional Review Boards and with informed consent of the patients. (2) BM (25–130 mL, and typically 50 mL) is taken from the posterior iliac crest, and hBMN cells are isolated from the BM by density gradient centrifugation. We might compare this amount (*i.e.*, 50 mL) with the 400–1000 mL of BM that is taken for the therapy of hematopoietic diseases with BM transplantation. (3) hBMN cells (typically $100\text{--}300 \times 10^6$ cells) are suspended into a small amount of solution (*e.g.*, 10 mL), and an intracoronary infusion of the hBMN cell solution is injected into the MI site of the patient at a few hours to 2 weeks after PCI therapy (typically 3–8 days after PCI). hBMN cells include 0.01% MS cells and 2–4% endothelial progenitor cells/hematopoietic stem cells, and most hBMN cells are consisted of hematopoietic mononuclear cells at various stages of maturation.^{6,50}

In early trials, such as the REPAIR-AMI trials³⁹ and BOOST,^{37,42–44,51} intracoronary autologous hBMN cell transfer enhanced several parameters of diastolic function in AMI patients. This recovered cardiac function can be elucidated by the function of CD34⁺ endothelial progenitor cells/hematopoietic stem cells in (1) growth factor secretion, (2) transdifferentiation into cardiomyocytes, endothelial cells, and smooth muscle cells *in vivo*, and (3) angiogenesis.^{6,52,53} However, transplantation of hematopoietic stem cells (CD133⁺CD34⁺ cells) was reported to enhance LVEF only minimally (2%). On the other hand, hBMN cell transplantation led to significant enhancement of LVEF (4–5%).⁵⁴

G-CSF (granulocyte colony-stimulating factor) is reported to mobilize hematopoietic stem cells from BM to peripheral blood. Then, some clinical trials on the treatment of AMI patients using G-CSF instead of intracoronary transfusion of hBMN cells were studied to investigate whether hematopoietic stem cells in hBMN cells play a critical function in improvements in AMI patients. However, G-CSF therapy exhibited almost no improvement in AMI patients in the clinical trials.^{52,55,56}

The timing of the intracoronary hBMN cell injection following PCI in AMI patients is extremely important. Late infusion (2–3 weeks after AMI, Late TIME trial³⁰) or early infusion of hBMN cells (less than 24 h, REGENERATE-AMI trial²³) was found not to enhance regional or global functions when the

AMI patients were evaluated at 6 months or 1 year after AMI. Infusion of intracoronary hBMN cells at 4–7 days after PCI or AMI should be the best timing for improvements in incidence of revascularization, end-systolic dimension, LV, and LVEF with this treatment.⁵⁷ On the other hand, Traverse and his colleagues reported that AMI patients who underwent infusion of intracoronary hBMN cells following PCI at 3 or 7 days after PCI showed recovery of global and regional LV function, which is similar to patients in the placebo group (Time trial). Therefore, effect of administration of intracoronary hBMN cells in AMI patients was not found in this trial.³¹

Dill and his colleagues reported that the intracoronary treatment of hBMN cells in AMI patients enhanced LVEF and improved entirely abrogated progressive end-systolic volume (ESV) expansion.⁴⁰

The mechanisms by which multiple cells derived from the BM function in cardiac repair remain unclear, although many experimental studies, clinical trials^{28,36,37,39,41–44,51,58} and meta-analyses^{54,57} have showed the benefits of hBMN cells on myocardial ischemia. Furthermore, some studies have shown that the intracoronary infusion of hBMN cells did not improve cardiac functions.^{36,46,59} In summary, intracoronary infusion of hBMN cells in AMI patients seems to induce a small enhancement in LVEF and has a restricted impact on left ventricular remodeling. However, infarct size is generally reduced by hBMN cell implantation.^{28,36,37,39,41–44,51,58}

Some other cell sources were used to cure AMI patients. Hirsch and his colleagues studied a randomized controlled trial to evaluate the effect of intracoronary infusion of mononuclear cells from BM and peripheral blood in AMI patients.²⁹ In this HEBE trial, the AMI patients processed with PCI had an intracoronary infusion of hBMN cells (69 patients) or peripheral blood mononuclear cells (66 patients) from 3 days to 8 days after AMI. No infusion of mononuclear cells to the patients as the standard therapy was also performed in this trial. Myocardial function as well as regional and global LV volumes were investigated by MRI (magnetic resonance imaging) before randomization and 4 months following treatment.²⁹ They reported no significant difference in LVEF or infarct size in these three groups.

6.2.2.3 MI Therapy Using CXCR4⁺CD34⁺ Progenitor Cells

There are heterogeneous populations of cells in hBMN cells, which are composed of committed granulocyte lineages, lymphocyte, and monocyte, as well as subpopulations of multipotent stem cells and progenitor cells. These cells are considered to work key roles in structural and functional recovery of the myocardium. However, the effects of intracoronary infusion of hBMN cells are still unknown after several clinical trials. Then, Tendera and his colleagues proceeded a clinical trial of “REGENT” using some populations of hBMN cells to evaluate whether some types of cells would facilitate the recovery of AMI patients.³⁵ They selected CXCR4 (chemokine receptor type 4) and CD34-expressing cells (CXCR4⁺CD34⁺ progenitor cells) using MACS after purification of hBMN cells from BM cells, because CXCR4 interacts

with SDF-1 (stromal cell-derived factor 1) in the myocardium. CXCR4⁺ cells may contribute to engraft, home, and mobilize to the ischemic myocardium, because AMI patients upregulated SDF-1 expression in the ischemic myocardium.⁶⁰ On the other hand, CD34⁺ cells are described as endothelial/hematopoietic progenitor cells. Two million of CXCR4⁺CD34⁺ progenitor cells were treated in an intracoronary infusion into patients 7 days after PCI. The cardiac function in three groups of patients were evaluated: (a) a control group that did not take cell therapy (forty patients), (b) a CXCR4⁺CD34⁺ progenitor cell-treated group (eighty patients), and (c) an unselected hBMN cell-treated group (eighty patients).³⁵

LVEF enhanced by 3% in AMI patients treated with CXCR4⁺CD34⁺ progenitor cells and hBMN cells, while no change in LVEF was reported in AMI patients in the control group after 6 months. A significant enhancement in LVEF was only observed in AMI patients treated with CXCR4⁺CD34⁺ progenitor cells or hBMN cells who had an LVEF of <37% before cell treatment.³⁵ No clinical difference was found among the CXCR4⁺CD34⁺ progenitor cell-treated group and hBMN cell-treated group in this study. However, the hBMN cell-treated group used only 50–70 mL of BM harvested, while the CXCR4⁺CD34⁺ progenitor cell-treated group used 100–120 mL of BM harvested in this study.³⁵ This evidence, along with the laborious sorting of specific cells using MACS, indicate that CXCR4⁺CD34⁺ cell treatment is not preferable compared to conventional hBMN cell treatment.

6.2.2.4 MI Therapy Using Allogeneic and Autologous HMS Cells

Autologous BM-derived hMS cells were also used to treat AMI patients. Lee and his colleagues studied a randomized pilot clinical trial to evaluate the efficacy and safety of autologous hMS cells in AMI patients.⁴⁷ Autologous hMS cells were obtained from following methods: BM (50 μ L) was aspirated from each patient. hBMN cells were isolated from BM by the density gradient centrifugation method using Histopaque-1077. hBMN cells were cultivated on tissue culture polystyrene (TCP) plates to remove non-adherent cells and proliferated for 4–5 passages; most of the cells attaching on the TCP plates were hMS cells. The AMI patients treated with autologous hMS cells exhibited an LVEF enhancement of 6%, while the AMI patients in the control group exhibited only a 1.6% enhancement in LVEF where these data were evaluated from SPECT (single-photon emission computed tomography) half year after treatment.⁴⁷ Then, this clinical trial indicates that intracoronary infusion of hMS cells derived from BM is safe, with modest enhancement in LVEF at 6-month follow-up evaluation.

Allogeneic but not autologous BM-derived hMS cells were also used to treat AMI patients. Hare and his colleagues studied a PROCYMAL clinical trial, which is a placebo-controlled, double-blind, dose-ranging (0.5, 1.6, and 5 million cells per kg) safety trial of intravenous allogeneic BM-derived hMS cells in reperfused patients with AMI. In this trial, hMS cells were implanted on days 1–10 after AMI (Figure 6.4A).³³ The patients treated with hMS cells

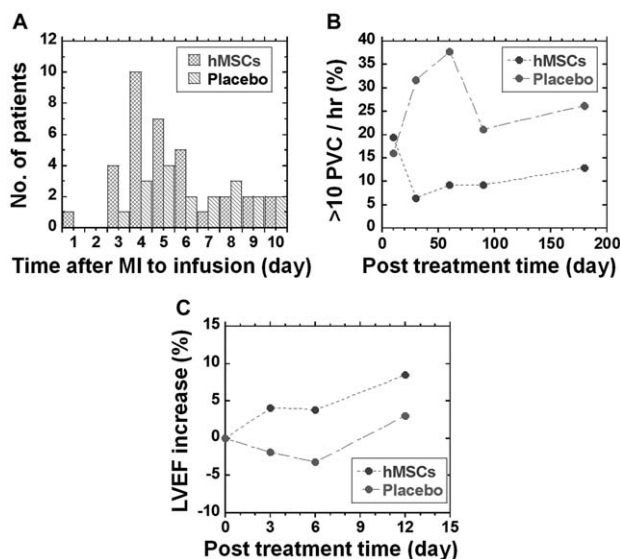


Figure 6.4 Intravenous infusion of allogeneic human bone marrow-derived MS cells in acute myocardial infarction. (A) Patient information at the time when human bone marrow-derived MS cells were infused after myocardial infarction. (B) Percentage of patients with a premature ventricular contraction (PVC) of >10 as a function of time after infusion of human bone marrow-derived MS cells or the placebo. (C) LVEF increase from baseline as a function of time after infusion of human bone marrow-derived MS cells or the placebo.³³ Adapted from ref. 33 with permission from the American College of Cardiology Foundation, Copyright 2009.

indicated less premature ventricular contraction (PVC) in comparison to AMI patients in the placebo control group on day 10 following hMS cell implantation (Figure 6.4B). Moreover, AMI patients treated with hMS cells exhibited enhanced LVEF and reverse remodeling of the heart (Figure 6.4C), while any improvement in cardiac function was observed for AMI patients in the placebo group, which were evaluated from cardiac MRI. Moreover, ambulatory electrocardiogram monitoring showed a reduction in ventricular tachycardia episodes in hMS cell-treated patients and pulmonary function test indicated an improvement in forced expiratory volume in 1 s.⁴⁷ This clinical trial showed the relatively excellent therapeutic effects of allogeneic hMS cells in AMI patients, as well as the safety of this process.

6.2.2.5 MI Therapy Using Autologous Cardiosphere-derived Cells and Cardiac Stem Cells

Autologous cardiac stem cells from BM were also used to cure patients with chronic heart disease. Autologous BM-derived cardiopoietic stem cells were implanted into patients endoventricularly in the C-CURE multicenter

randomized trial.²⁶ The cardiopoietic stem cells were obtained from the following processes: hBMN cells were obtained from BM using the conventional centrifugation method. Subsequently, hBMN cells were cultivated on TCP plates for one passage and then cultivated in media including a cardiogenic cocktail (α -thrombin, cardiotrophin), FGF-2 (fibroblast growth factor 2), activin A, bone morphogenetic protein 4 [BMP 4], and TGF- β (transforming growth factor- β) for three passages. Of the differentiated cells, a minimum of 85% of cells expressed a more than two-fold of myocyte enhancer factor 2C (MEF2c) that is the gene included in cardiac morphogenesis, in the nucleus *versus* the cytosol.²⁶ The cardiopoietic stem cells were implanted into mapped sites at one min per injection, with an average of eighteen injections per one patient (Figure 6.5), with the cells delivered into the site of dysfunctional but viable myocardium evaluated by a voltage of >4 mV (the unipolar map in Figure 6.5) with decreased longitudinal linear shortening (Figure 6.5).²⁶

In this trial, LVEF increased from 28% to 35% with implantation of autologous cardiopoietic stem cells, while patients who were not processed with the cells exhibited no enhancement in LVEF.²⁶ The decrease in LV ESV was also

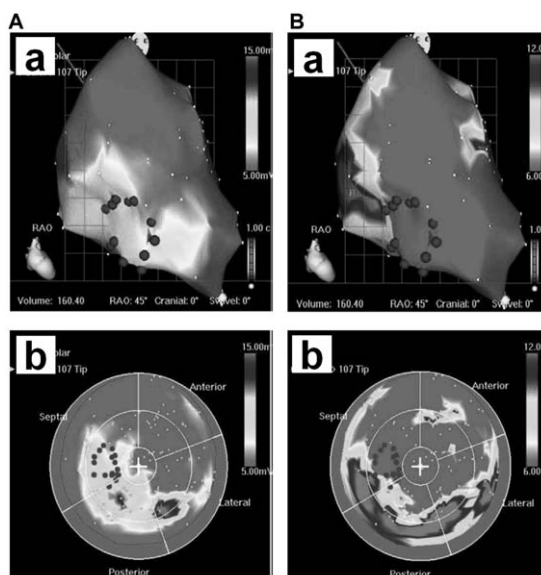


Figure 6.5 Stem cell delivery evaluated by intramyocardial mapping and navigation. (A, B) Endomyocardial delivery of bone marrow-derived cardiopoietic stem cells using electromechanical guidance in a patient enrolled in the C-CURE trial. The upper illustrations indicate a right anterior oblique projection of unipolar voltage (A(a)) and linear local shortening maps (LLS, B(a)). A bulls-eye view of mechanical and electrical maps with unipolar voltage (A(b)) and linear local shortening (B(b)). Stem cells were implanted (spots) into sites of dysfunctional yet viable myocardium analyzed by a voltage of >4 mV with reduced LLS.²⁶ Adapted from ref. 26 with permission from the American College of Cardiology Foundation, Copyright 2009.

observed widely in the group processed with autologous cardiac stem cells in comparison to the group of patients who were not processed with cardiopoietic stem cells. This trial indicated that cardiopoietic stem cell therapies are safe and feasible, with symptoms of benefit for AMI patients with chronic heart failure.

Makkar and his colleagues investigated usage of CDCs (cardiosphere-derived cells) for the treatment of patients 14–28 days after MI (CADUCEUS clinical trial).²⁷ CDCs were obtained from the following processes (Figure 6.6):²⁷ Approximately 280 mg of endomyocardial tissue was taken

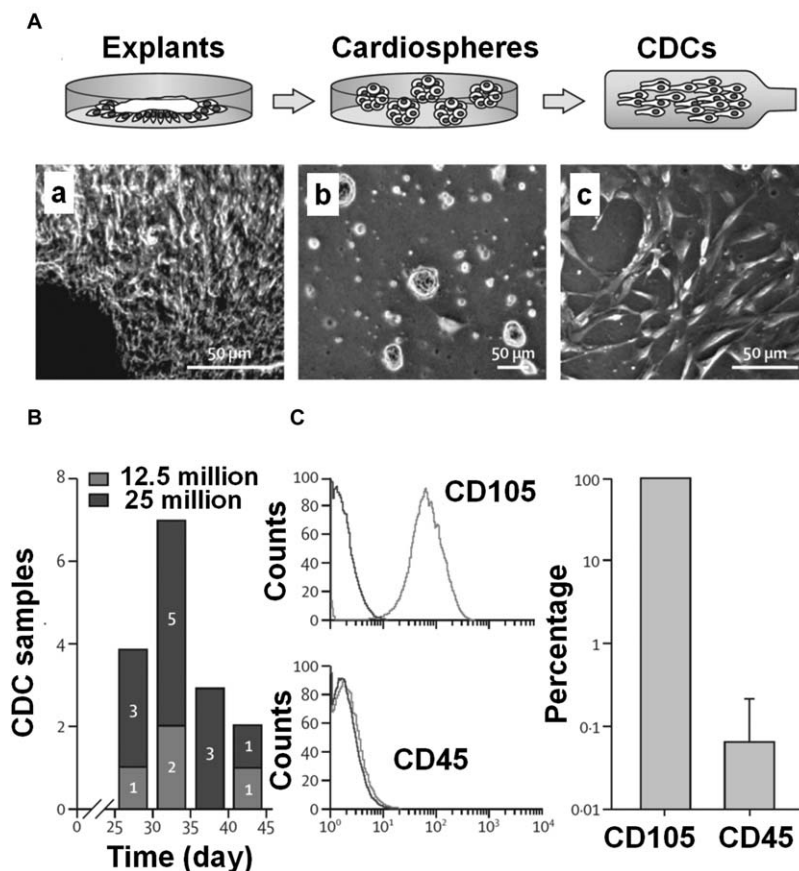


Figure 6.6 Preparation and properties of CDCs (cardiosphere-derived cells). (A) Preparation of CDCs. Biopsy samples were cut into small pieces of explants (a), which spontaneously enhanced the cell population. The explants were cultivated in a suspension to prepare 3D cardiospheres (b). Then, the cardiospheres attached on TCP plates and generated CDCs (c). (B) Histogram of the time required to prepare a prespecified dose of 12 500 000 or 25 000 000 cells. (C) Expression of CD45 and CD105 CDCs evaluated by flow cytometry. Most CDCs showed expression CD105 (98%), while less than 0.5% of CDCs showed expression of CD45.²⁷ Adapted from ref. 27 with permission from Elsevier, Copyright 2012.

from the patient by biopsy. The tissue samples were minced into approximately 1-mm explants, and the explants were cultivated on TCP plates. This process was almost the same to the typical process for preparation of hADS cells from fat tissue samples.^{61,62} The adherent cells were harvested and subsequently cultivated in suspension to prepare 3D (three-dimensional) cardiospheres. Subsequently, the cardiospheres were cultivated on TCP plates to prepare CDCs.²⁷ CDCs were cultivated for 2–5 passages to generate the expected dose of CDCs within 5 weeks. Of the CDCs, 95% showed CD105 expression (*i.e.*, CD105⁺ cells), while less than 5% of CDCs were CD45⁺. Autologous CDCs (25 000 or 12 500 000 cells) were administered an intra-coronary infusion into the infarct-related artery of the patients 45–90 days after MI in patients who showed a 39% mean LVEF and 24% scar in their hearts. Major adverse cardiac events did not occur in the patients in the CDC-infusion group and control group.²⁷ Moreover, cardiac tumors did not develop and no patients were found to die in either group, which indicate that CDC implantation is safe. Cardiac scar tissue was greatly decreased and new healthy tissue was formed after infusion with CDCs in this clinical trial (CADUCEUS) (Figure 6.7).²⁷ However, in this trial, they did not find any improvement in endo-systolic volume, end-diastolic volume, or LVEF with CDC infusion relative to that in the control group. This might be explained from the reason of the extremely low survival rate of implanted cells *in vivo* and relatively small numbers of cells implanted. It might be a good idea that CDC implantation with injectable hydrogels into the infarct-related artery of the patients would lead to enhanced cardiac function after the treatment.

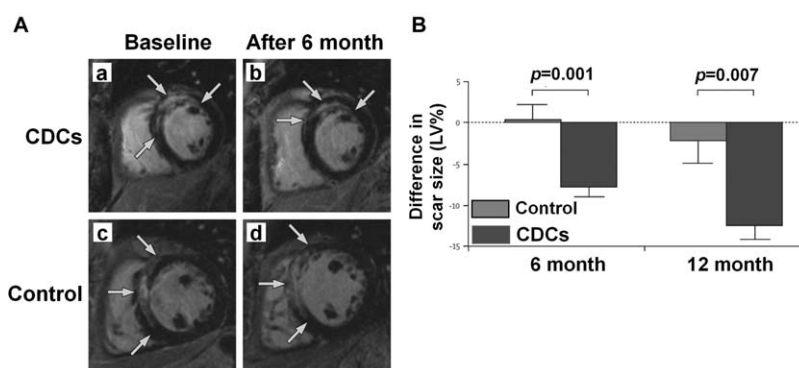


Figure 6.7 Representative MRI of hearts of patients with myocardial infarction and changes in scar sizes in the CADUCEUS clinical trial. (A) Short-axis MRI of the heart of a patient at baseline (82 days after myocardial infarction, (a)) and 180 days after CDC implantation (b). Short-axis MRI of the heart of a patient at baseline (77 days after myocardial infarction, (c)) and after 180 days (d) in a control. Infarct scar tissue (arrows) shows as sites of hyperintensity and viable myocardium is marked by spots. (B) Difference in scar size (LV%) from baseline to 180 days or 1 year after treatment.²⁷ Adapted from ref. 27 with permission from Elsevier, Copyright 2012.

Autologous CSCs (cardiac stem cells, lineage-negative cells and c-kit⁺) were also used to cure patients with ischemic cardiomyopathy in SCPIO clinical trial.³² CSCs were obtained from the following processes: The right atrial appendage was isolated during CABG. CSCs were also purified using a method that is the same as that used to isolate hADS cells from fat tissue.^{61,62} In brief, the atrial tissue was cut into small pieces (<1 mm³) and suspended in cell culture media. After collagenase digestion of the tissue, the cells were inoculated on TCP plates and cultivated for several passages to purify CSCs. In general, only stem cells are attached and proliferate on TCP plates. Then, the cells were isolated using MACS that targets c-kit⁺ (c-kit-expressing) cells. One million CSCs were implanted into anterior wall infarct, and 0.5 million cells were implanted into infarct in the right coronary artery or left circumflex.³²

LVEFs recovered to 38.5% from 30.3% before CSC infusion at 4 months after implantation of CSCs, while the LVEFs of patients in the control group show no change (30.1%) at 4 months after CABG.³² This clinical trial indicates that intracoronary infusion of autologous CSCs seems to be a reasonable and reliable approach to reduce infarct size and to enhance recovery of LV systolic function after MI. On the other hand, the effect of implantation of CDCs²⁷ and CSCs (c-kit⁺ cells)³² on the treatment of the patients with MI is currently found to be controversial. The effect of implantation of c-kit⁺ cardiac stem cell is typically regarded as a paracrine effect of c-kit⁺ cardiac stem cell.^{63,64} Moreover, it should be commented that the isolation and purification of CSCs is not easy in comparison to the isolation and purification of stem cells derived from adipose tissue or BM from a bioengineering point of view. It is important to design much sophisticated processes to isolate CDCs, cardiopoietic stem cells, and CSCs from tissues in future.

6.2.2.6 MI Therapy Using Fetal Stem Cells

The hWJ-MS cells (human Wharton's jelly-derived mesenchymal stem cells) could be isolated from a continuum from the sub-amnion to the perivascular region of umbilical cord.^{65,66} hWJ-MS cells shows several typical marker expression of hMS cells and hES cells in early passages.⁶⁶ Moreover, hWJ-MS cells can induce differentiation into cardiomyocytes and endothelial cells.⁶⁷⁻⁶⁹ Therefore, Gao and his colleagues studied the effect of intracoronary implantation of hWJ-MS cells in AMI patients. In this trial, 116 AMI patients were randomly selected to take a placebo or hWJ-MS cells in their infarct arteries at 5–7 days after PCI.⁴⁸ The absolute enhancement in myocardial viability evaluated by PET (positron emission computed tomography) and perfusion within the infarcted site analyzed by SPECT indicated hugely better outcomes in the patients implanted with hWJ-MS cells than in the patients who received the placebo at 4 months after treatment. The enhancement in the LVEF of patients implanted with hWJ-MS cells (8%) was found to be much higher than that of patients in the placebo group (3%) after 18 months (Figure 6.8A). Moreover, reduction in LV ESVs and end-diastolic volumes of

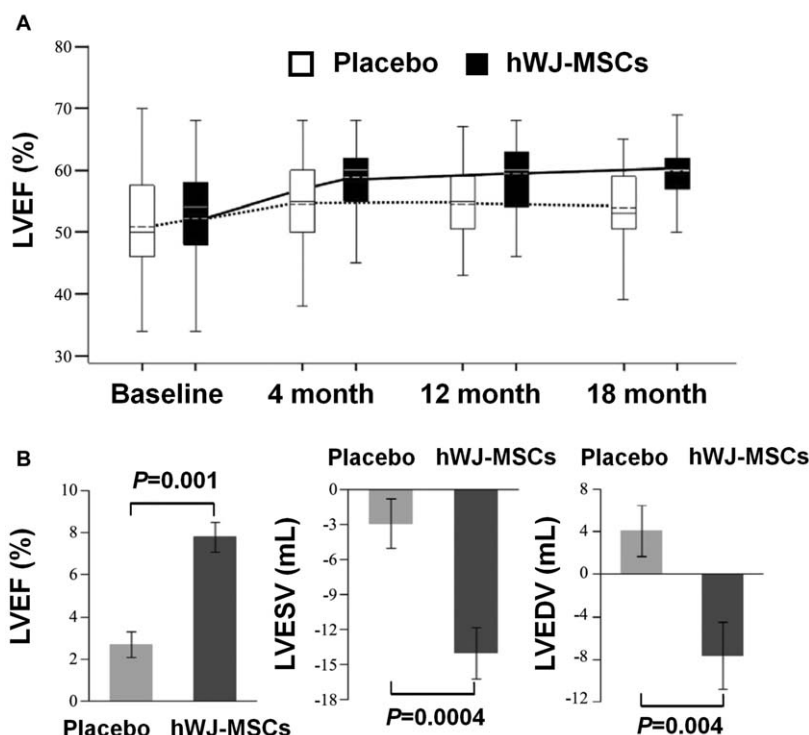


Figure 6.8 Intracoronary infusion of Wharton's jelly-derived MSC cells in acute myocardial infarction. (A) Comparison of changes in global LVEF between the hWJ-MSC (Wharton's jelly-derived MSC) treatment group and the placebo group analyzed by 2D echocardiography after infusion and before infusion at 4 months, 1 year, and 18 months. (B) Absolute changes in LVEF, LVESV (left ventricular end-systolic volume), and LVEDV (left ventricular end-diastolic volume), between the WJ-MS cell (hWJ-MSCs) group and the placebo group at 18 months evaluated by echocardiograph.⁴⁸ Adapted from ref. 48, <https://dx.doi.org/10.1186%2F12916-015-0399-z>, under the terms of the CC BY 4.0 license, <https://creativecommons.org/licenses/by/4.0/>.

patients treated with hWJ-MS cells relative to those of patients in placebo group were found after 18 months (Figure 6.8B).⁴⁸ This clinical trial indicates that the intracoronary implantation of hWJ-MS cells is safe and suggests another option in the treatment of AMI patients.

6.2.2.7 MI Therapy Using Adipose-derived Stem (ADS) Cells

Adipose tissue includes multipotent ADS cells, which represent an abundant and easily accessible cell source. ADS cells could induce differentiation into endothelial cells and cardiomyocytes *in vivo* in animal studies that suggest that ADS cells might be another attractive cell source to generate new blood vessels and cardiomyocytes in patients with chronic ischemic heart

disease.⁷⁰ There are some clinical trials using freshly purified human ADS cells (APOLLO clinical trial [NCT00442806] and PRECISE clinical trial [NCT00426868]) or cultivated and expanded ADS cells (MyStromalCell clinical trial [NCT01449032]) to cure AMI patients. However, no reports from these clinical trials can be obtained until now.

The double-blinded, randomized, and placebo-controlled trials of intracoronary infusions of some cell types, including (1) hPS cell-derived cardiomyocytes, (2) hPS cell-derived human cardiac progenitor cells, (3) hADS cells, and (4) human BM-derived MS cells to cure AMI patients are necessary to be investigated more and we should compare the results of the improved LV function in AMI patients after each treatment in the future.

6.2.3 Future Trends of MI Therapy Using Stem Cells

In current clinical trials using hES cells, the differentiated cells into progenitor cells (*e.g.*, cardiac progenitors) are typically used, while hiPS or hES cells are sophisticatedly induced to differentiate into not progenitor cells, but mature stages of desired lineages of the cells in typical basic studies.^{71–74} Furthermore, hMS cells, which are not induced to differentiate into cardiac lineages or non-purified mononuclear cells from BM are widely used in the clinical trials of AMI patients,^{24–45,47,49,75} while researchers investigate the differentiation of hMS or hPS cells into mature cardiomyocytes in typical basic studies.^{76–80} Only a few trials have been reported to date using hPS cells (*i.e.*, hES cells).^{17,18} Therefore, we find a gap between clinical use of hMS cells or hPS cells and fundamental studies on cultivation and differentiation protocols for stem cells. The author suggests that bioengineers will try to reduce these gaps between clinical application and fundamental studies of hPS cells and hMS cells after consideration of this book. It would be valuable to design cell sorting biomaterials for the culture of cells on specific materials for the isolation of desired cells, which we are intended to purify or differentiate.⁸¹ The sophistication of protocols for implanting stem cell-derived cells or stem cells into patients should be considered with the usage of injectable hydrogels or scaffolds for better survival rates of implanted cells in patients over a long period of time.

6.3 Stem Cell Therapy for Macular Degeneration Disease in Clinical Trials

Present clinical trials, which investigate human pluripotent stem cell (hPS cell)-based therapy extensively target treatment of macular degeneration of the eyes, because the eyes are isolated from other tissues and generate a low immunoreaction in nature. We consider current bioengineering approaches and material use in combination with stem cell therapies for macular degeneration disease treatment in this chapter. RPE (retinal pigment epithelium) induced from hPS cells is widely used in common clinical trials

for the treatment of patients, whereas BMN cells or MS cells are intravitreally implanted into the eyes of patients. We further evaluate reported negative effects of stem cell therapies, such as patients being blind by implantation of hADS cells, which have been widely used in “stem cell clinics”.

The clinical trials for stem cell therapy using hPS cells (mainly hES cells) have been reported only for four major diseases from the ClinicalTrials.gov database investigation: (1) spinal cord injury, (2) diabetes, (3) acute myocardial infarction (AMI), and (4) macular degeneration [**Stargardt’s macular dystrophy** and **age-related macular degeneration (AMD)**] (Table 6.2). Ocular disease treatment is the first target for the human trials using hES cells, and phase I/II trials indicate some possible efficacy with promising safety results.^{13,14} The present clinical trials of hPS cell-based therapies extensively focus on treatment of macular degeneration in the eye (Table 6.2). Macular degeneration is considered because the eye tissue is isolated from other tissues and has a minimum immuno-response in nature.^{5,82} It is able to visualize the internal tissues *via* the lens after implantation of the cells. AMD and Stargardt’s macular dystrophy are the progressive degradation of light-sensing photoreceptor cells and their supportive RPE. Several clinical trials are now ongoing for hPS cell-derived RPE for AMD and Stargardt’s macular dystrophy (Table 6.2).^{12–14,82–84}

Currently, some clinical trials using hPS cells have been performed, and there are some excellent reviews, which describe the present status of cell therapies using hPS cells.^{1–4,10,85,86} However, these review articles do not account for bioengineering techniques, such as the implantation protocol, hPS cell cultivation protocol, and status and condition of hPS cells, especially with regard to monolayer or suspension usage without or with materials (hydrogels, membranes, or scaffolds) at the injected sites. We discuss present bioengineering aspects and material use of stem cell therapies using hPS cells for the treatment of macular degeneration diseases in this chapter. Moreover, we consider clinical trials using human fetal or adult stem cells such as hMS cells that are targeted for the treatment of macular degeneration. Recently, Zarbin wrote an excellent review for cell-based therapies on degenerative retinal diseases from cell biological aspects.⁸⁵ Zarbin’s review will help readers to study cell biological aspects of stem cell therapies for macular degeneration diseases.

6.3.1 Macular Degeneration Diseases and Eye Structure

The eye has three tissue layers: the outermost layer (sclera), the middle vascularized layer (choroid), and the innermost layer (retina; inset), (Figure 6.9A).⁸¹ The outermost region of the retina is the location of the RPE, which maintains photoreceptor health by transporting nutrients and recycling shed photoreceptor parts. The RPE is a monolayer of pigmented epithelial cells, which is positioned between the neural retina and Bruch’s membrane.⁵ There are three phases of neurons in the interior of the eye within the RPE: a middle layer of connecting neurons (bipolar, horizontal,

Table 6.2 Clinical trials for cure of patients with macular dystrophy using hES and hiPS cells. (ClinicalTrials.gov).⁸¹ Adapted from ref. 81 with permission from Elsevier, Copyright 2017.^a

Differentiated cells	Disease for treatment	Cell no. transplanted	Cell delivery method	Phase of trial	ClinicalTrials.gov identifier	Country (company or institute)	Period
hESC-derived cardiac progenitor cells	Heart failure (coronary artery disease)	Not specified	Cells in fibrin gel patch	Phase I	NCT02057900	France (Hôpitaux de Paris)	2013–2018
hESC-derived RPE	Advanced dry AMD	10 ⁵ cells	Cells on Parylene-C film	Phase I/II	NCT02590692	USA (Regenerative Patch Technologies)	2015–2022
RPE	SMD	Not specified	Not specified	Phase I	NCT01625559	Korea	2012–2015
hESC-derived RPE	AMD and SMD	10 ⁵ cells	Cell suspension or cells on polymeric substrate	Phase I/II	NCT02903576	Brazil (Federal Univ. of São Paulo)	2015–2017
hESC-derived RPE	AMD	Not described	Not described (cell suspension?)	Phase 0	NCT02755428	China (Chinese Academy of Sciences)	2015–2016
hESC (MA-09)-derived RPE	Advanced dry AMD	5, 10, 15, or 20×10 ⁴ cells	Not described (cell suspension?)	Phase I/II	NCT01674829	Korea (CHABiotech)	2012–2016
hESC (MA-09)-derived RPE	SMD	5, 10, 15, or 20×10 ⁴ cells	Cell suspension	Phase I/II	NCT01469832	UK (Ocata Therapeutics)	2011–2015
hESC (MA-09)-derived RPE	SMD	5, 10, 15, or 20×10 ⁴ cells	Cell suspension	Phase I/II	NCT02445612	USA (Ocata Therapeutics)	2012–2029
hESC (MA-09)-derived RPE	SMD	5, 10, 15, or 20×10 ⁴ cells	Cell suspension	Phase I/II	NCT01345006	USA (Ocata Therapeutics)	2011–2015
hESC (MA-09)-derived RPE	Advanced dry AMD	5, 10, 15, or 20×10 ⁴ cells	Cell suspension	Phase I/II	NCT02463344	USA (Ocata Therapeutics)	2012–2029
hESC (MA-09)-derived RPE	Advanced dry AMD	5, 10, 15, or 20×10 ⁴ cells	Cell suspension	Phase I/II	NCT01344993	USA (Ocata Therapeutics)	2011–2015
hESC-derived RPE	Advanced dry AMD	5×10 ⁴ –10 ⁵ cells	Cell suspension	Phase I/II	NCT02286089	Israel (Hadassah Ein Kerem Univ. Hospital)	2015–2018
hESC (MA-09)-derived RPE	SMD	5×10 ⁴ cells	Cell suspension	Phase I	NCT01625559	Korea (CHABiotech)	2012–2015

hESC-derived RPE (PF-05206388)	Acute wet AMD	Confluent cells on membrane	Cells on polyester membrane (6×3 mm)	Phase I	NCT01691261	UK (Pfizer)	2015–2016
hESC-derived RPE	AMD and SMD	Not specified	Cell suspension	Phase I	NCT02749734	China (Southwest Hospital)	2015–2017
hESC-derived RPE	Stargardt's macular dystrophy treatment	5, 10, 15, or 20×10 ⁴ cells	Cell suspension	Phase I/II	NCT02941991	UK (Astellas Institute)	2013–2019
hiPSC-derived RPE	Wet AMD	Not specified	Cell sheet	Phase I		Japan (Kyoto Univ.)	On hold
hESC-derived pancreatic endoderm cells (PEC-01)	Type 1 diabetes mellitus	Not specified	Encapsulated cells in medical device (VC-01, PEC-Encap)	Phase I/II	NCT02239354	USA (ViaCyte)	2014–2017
hESC-derived pancreatic endoderm cells (PEC-01)	Type 1 diabetes mellitus	Not specified	Encapsulated cells in medical device (VC-01, PEC-Encap)	Not specified	NCT02939118	USA (ViaCyte)	2016–2023
hESC-derived pancreatic endoderm cells (PEC-01)	Gestational diabetes Type 2 Diabetes Glucose intolerance	Not specified	Encapsulated cells in medical device (VC-01, PEC-Encap)	Not specified	NCT01839448	USA (ViaCyte)	2014–2017
hESC-derived OPCs (AST-OPC1 cells)	Spinal cord injury	One or two injections of 2 or 10×10 ⁶ cells	Cell suspension	Phase I/II	NCT02302157	USA (Asterias Biotherapeutics)	2015–2018
hESC-derived OPCs (GRNOPC1 cells)	Spinal cord injury	2 million cells	Cell suspension	Phase I	NCT01217008	USA (Asterias Biotherapeutics)	2010–2013

^aAMD, age-related macular degeneration; OPC, oligodendrocyte progenitor cells; RPE, retinal pigment epithelium; SMD, Stargardt's macular dystrophy

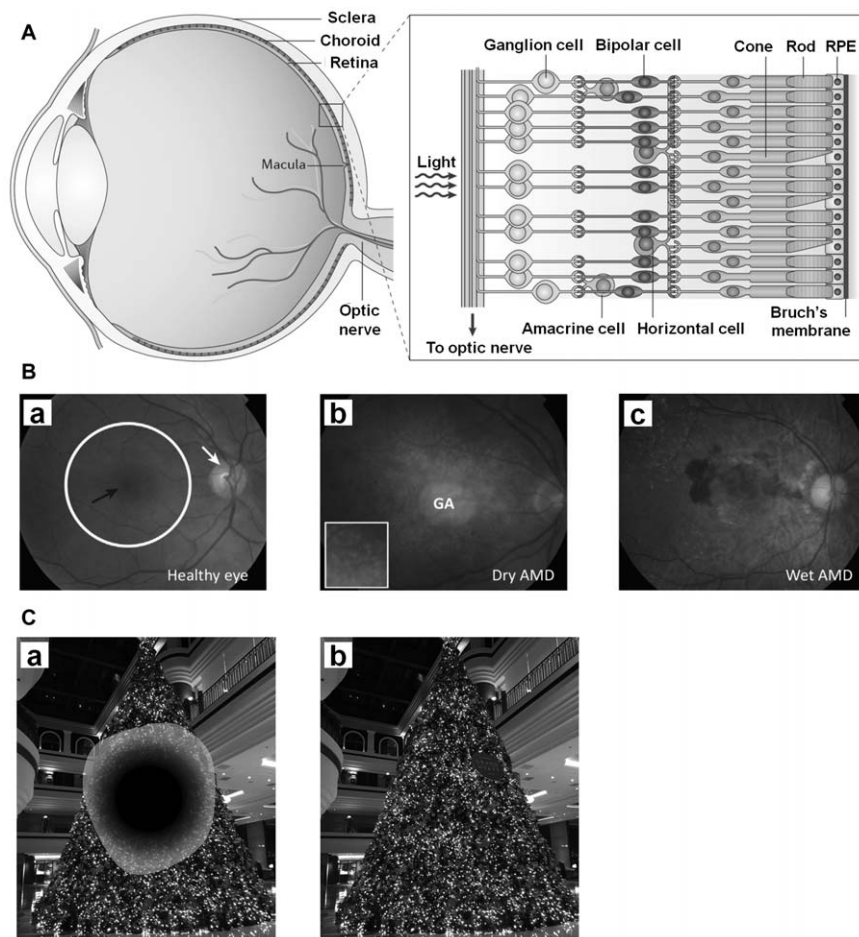


Figure 6.9 Eye structure and AMD. (A) Eye structure.⁵ Copyright 2015. Adapted with permission from Springer Nature. (B) Fundus photographs of the eye from subjects with (a) normal macula (circle) and healthy fovea (arrow). The arrow shows the optic nerve. (b) Late-stage dry AMD. (c) Late-stage wet AMD (choroidal neovascularization).¹ Copyright 2013. Adapted with permission from Cell Press. (C) A simulated photo of vision in patients with late-stage AMD (a) and normal vision (b).⁸¹ Adapted from ref. 81 with permission from Elsevier, Copyright 2017.

and amacrine cells), photoreceptors of light-sensing rods and cones, and the innermost layer of ganglion cells that accept signals formed in the photoreceptor cells *via* the optic nerve and transfer the signals into the brain.⁵ The photoreceptor cells, ganglion cells, and RPE are able to be induced from hES and hiPS cells for stem cell therapies and disease modelling.

The earliest signals of degeneration in the macula are the appearance of drusen (Figure 6.9B) that are abnormal accumulation of extracellular materials (proteins and lipids), which are generated between the RPE and

Bruch's membrane.⁸¹ AMD is classified into two kinds at the late stage, wet AMD and dry AMD. Dry AMD is a progressing disease slowly, which makes the gradual loss in central vision (Figure 6.9C). The majority (90%) of patients with AMD are classified as having dry AMD. Currently, no treatment has been developed for dry AMD.¹

A reduction in the RPE structural integrity makes the growth of new blood vessel in the subretinal space. These leaky vessels form blood (fluid) to store in the subretinal region. The accumulation of blood (fluid) at the macula in wet AMD distorts the natural configuration of the macula (Figure 6.9B(c)), which generates a deterioration and irreversible scarring in the central vision of the patients. Only palliative treatment is taken for the patient with wet AMD, for example, intraocular injection of angiogenesis inhibitors (Lucentis, Avastin, or Eylea).

6.3.2 Bioengineering in Stem Cell Therapies for Macular Degeneration Diseases

Pluripotent stem cells (hES cells and hiESCs) as well as fetal or adult stem cells, such as human adipose-derived stem (hADS) cells, human bone marrow stem (hBMS) cells, umbilical cord stem cells, neural stem cells from human fetal forebrain tissue, and RPE stem cells isolated from donor eyes, are used for stem cell therapies for macular degeneration diseases. Human trials using adult stem cells, fetal stem cells, hES cells, and hiPS cells for the administration of patients with macular degeneration diseases are exhibited in Tables 6.2 and 6.3. General stories of stem cell therapies for macular degeneration diseases are described in Figure 6.10. Differentiated cells, such as RPE cells, can be used for hiPS cells and hES cell therapies (Figure 6.10). This selection is made to avoid tumor creation from implanted and undifferentiated hES cells and hiPS cells. On the other hand, fetal or adult stem cells are implanted into the subretinal region of patients with no induction into desired cells such as photoreceptor cells or RPE (Figure 6.10).

Xeno-free and GMP grade culture and differentiation of hES cells and hiPS cells are demanded in the bioengineering aspects for stem cell therapy using hES cells and hiPS cells, while GMP grade and xeno-free purification and the cultivation of fetal or adult stem cells are important for stem cell therapy. The development of optimal cell cultivation materials for the proliferation of hES cells and hiPS cells and induction into photoreceptor cells or RPE cells should be interesting topics. Although cell culture plates coated with laminin-521, laminin-511, or recombinant vitronectin are reported to maintain long-term cultivation of hES cells and hiPS cells,^{1,87} appropriate cell cultivation materials (plates) for the induction of hES cells and hiPS cells into desired lineages of the cells are currently not identified.⁷⁴ In purification of adult stem cells such as hBMS cells, the fraction of mononuclear cells (hBMN cells) is purified from BM by the density gradient method, and then, hBMN cells are cultured on TCP plates for 1–3 passages to prepare hBMS cells. The sorting mechanism of hBMS cells is based on high adhesive

Table 6.3 Clinical trials for cure of patients with macular dystrophy using human fetal stem cells and adult stem cells (ClinicalTrials.gov).⁸¹
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Cell source	Disease for treatment	Cell no. transplanted	Cell delivery method	Phase of trial	ClinicalTrials.gov identifier	Country (company or institute)	Period
Autologous hBMSCs	AMD and SMD	10 ⁷ cells	Cell suspension (intravitreal injection)	Phase I/II	NCT01518127	Brazil (University of Sao Paulo)	2011–2015
Autologous hADSCs	Drt AMD	Unknown	Cell suspension (intravitreal injection)	None	NCT02024269	USA (Bioheart, Inc.)	2013–2017
hCNS-CSs	AMD	Unknown	Cell suspension (subretinal transplantation)	Phase I/II	NCT02137915	USA (StemCells, Inc.)	2014–2016
hCNS-CSs	AMD	10 ⁶ cells	Cell suspension (subretinal transplantation)	Phase I/II	NCT01632527	USA (StemCells, Inc.)	2012–2015
Autologous BM-CD34 ⁺ cells	AMD	Unknown	Cell suspension (intravitreal injection)	Phase I	NCT01736059	USA (UC Davis)	2012–2017
Autologous hBMSCs	MD	Unknown	Cell suspension (Retrolbulbar, subtenon, intravenous, intravitreal, and/or intraocular injection)	None	NCT01920867	USA (Retina Associates of South Florida)	2013–2017
hCNS-SCs	AMD	Unknown	Cell suspension (subretinal transplantation)	Phase II	NCT02467634	USA (StemCells, Inc.)	2015–2016
Autologous BMSCs	Dry AMD	Unknown	Cell suspension (intravitreal injection)	Phase I/II	NCT02016508	Egypt (Al-Azhar University)	2013–2015
UC-MSCs (CNT02476)	AMD	6, 12, 30, or 56 × 10 ⁴	Cell suspension (subretinal transplantation)	Phase I/II	NCT01226628	USA (Janssen Research & Development, LLC)	2010–2019
Autologous hBMSCs	AMD	Unknown	Cell suspension (Retrolbulbar, subtenon, intravenous, intravitreal and/or intraocular injection)	None	NCT03011541	USA (MD Stem Cells)	2016–2021
Autologous hBMSCs	Retinitis pigmentosa	10 ⁷ cells	Cell suspension (intravitreal injection)	Phase II	NCT01560715	Brazil (University of Sao Paulo)	2011–2013
hBMSCs	Retinitis pigmentosa	10 ⁶ cells	Cell suspension (intravitreal injection)	Phase I	NCT01531348	Thailand (Mahidol University)	2012–2014
hRPCs	Retinitis pigmentosa	0.5–3 × 10 ⁶ cells	Cell suspension (intravitreal injection)	Phase I/II	NCT02320812	USA (jCyte, Inc)	2015–2017

^ahCNS-SCs, human central nervous system stem cells; hRPCs, human retinal progenitor cells; UC-MSCs, human umbilical tissue-derived cells.

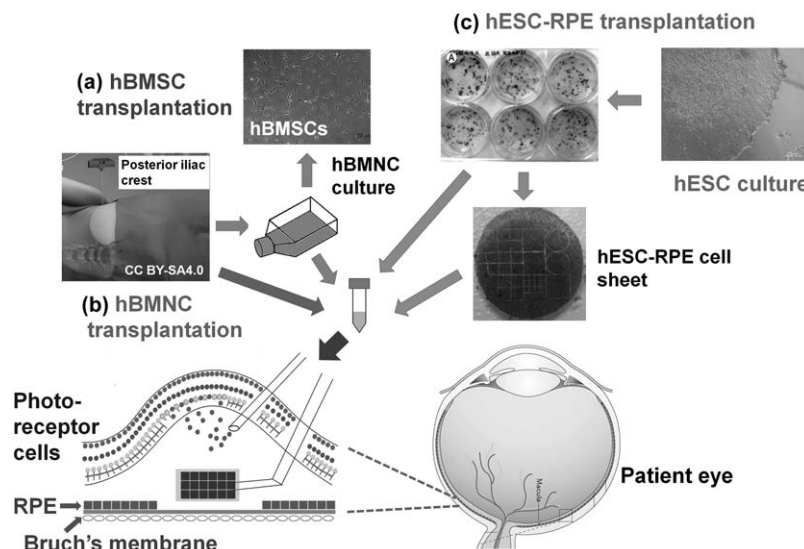


Figure 6.10 Illustration of stem cell therapy for macular degeneration diseases. (a) hBMN cells were sorted from bone marrow, and the bone marrow was taken from the posterior iliac crest and subsequently implanted into the subretinal region. (b) hBMS cells were purified by the culture of hBMN cells on TCP plates and subsequently implanted into the subretinal region. (c) hPS cells were induced into photoreceptor cells or RPE cells and subsequently implanted into the subretinal region. Photoreceptor cells or hPS cell-derived RPE cells were injected as a cell sheet or a single cell solution without and with materials (gels, membranes, or scaffolds).⁸¹

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properties of adult stem cells on TCP plates. A faster isolation method of hADS cells or hBMS cells without utilizing antibodies, such as MACS or FACS (fluorescence-activated cell sorting), is demanded from a bioengineering aspect. Cell sorting plates, which select desired cell types,^{88,89} or filtration method *via* porous polymer membranes that purify desired cell types using affinity to pore size of the membranes and/or the membrane materials,^{61,62,90} would be valuable for this purpose. At this moment, it is hard to discuss which stem cell types are preferable for stem cell therapies for macular degeneration diseases.

6.3.3 Biomaterial Assists in the Therapies for Macular Degeneration Diseases

From the bioengineering aspect of RPE implantation for macular degeneration disease therapies, the RPE patches with a supporting layer would be considered to be more valuable when compared with the infusion of a single

RPE cell suspension that is currently selected in typical clinical trials (Tables 6.2 and 6.3). The difference generates because the dissociation of RPE cells may guide dedifferentiation.¹ Furthermore, the remaining transplanted hES cell-RPE cells on the implanted region of the patients is extremely low, and it is hard to analyze the efficacy of the remaining cells. Moreover, Hsiung and colleagues reported that the polarized hES cell-derived RPE monolayers that were transplanted in the subretinal site of patient eyes with AMD had potentially high survival potential in comparison to hES cell-derived RPE cells transplanted in suspension.⁹¹ Therefore, several investigators have considered to develop a patch (cell sheet) of retinal progenitors, RPE, or hES cell-RPE cells with a supporting polymer material, although the patch of hES cell-RPE cells on a polymer material has been used for only one case in clinical application. Most of the investigations using the patch of hES cell-RPE cells are investigated for the treatment of macular degeneration diseases in preclinical researches (model animal experiments). Several polymeric materials have been developed for this purpose: polyamide nanofibers,¹ poly(ϵ -caprolactone),⁹² poly(glycerol sebacate) scaffold,⁹³ polyester membrane,⁹⁴ Parylene-C membrane,^{95–97} plasma polymers,⁹⁸ modified polytetrafluoroethylene,⁹⁸ polyimide membrane,⁹⁹ poly(methyl methacrylate) scaffold,¹⁰⁰ gelatin scaffold,¹⁰¹ and collagen gel.¹⁵ These polymeric materials are shown in Table 6.4. These polymeric materials work similar to artificial Bruch's membranes that support RPE and provides nutrients to RPE cells. The artificial Bruch's membranes must hold the following two important properties: nutrients should pass through the polymeric materials, and hES cell-derived RPE cells should attach and expand on the polymeric materials.⁹⁶

Parylene-C is a biostable polymeric material (Figure 6.11A), which has been used in several biomedical devices such as electrodes working as neural prostheses, pacemakers, and stents.³ Then, hES cell-derived RPE cells

Table 6.4 Polymeric biomaterials used for cultivation of retinal progenitors, RPE cells, hESCs-RPE cells to create cell sheets.⁸¹ Adapted from ref. 81 with permission from Elsevier, Copyright 2017.^a

Polymeric material	Morphology of materials	Biodegradability	Ref. (year)
Collagen	Hydrogel	Biodegradable	15 (2014)
Gelatin	Hydrogel (scaffold)	Biodegradable	101 (2014)
PMMA	Membrane	Non-biodegradable	100 (2007)
Polyimide	Membrane	Non-biodegradable	99 (2012)
PTFE	Film	Non-biodegradable	98 (2012)
Parylene-C	Membrane	Non-biodegradable	95–97 (2012–2013)
Polyester	Membrane	Non-biodegradable	94 (2012)
Poly(glycerol sebacate)	Membrane	Non-biodegradable	93 (2009)
Poly(ϵ -caprolactone)	Film	Non-biodegradable	92 (2008)
Polyamide	Nanofiber	Non-biodegradable	1 (2013)

^aPLGA, poly(D,L-lactic-co-glycolic acid); PLL, poly(L-lactic acid); PMMA, polymethylmethacrylate; PTFE, polytetrafluoroethylene.

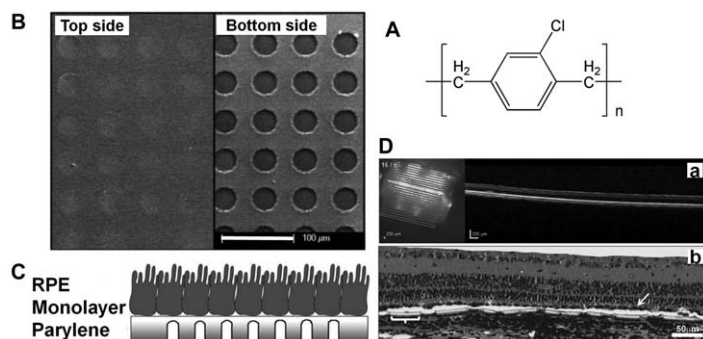


Figure 6.11 Transplantation of hES cell-derived RPE sheets supported on an ultrathin Parylene-C membrane in the porcine eye at the subretinal region. (A) Chemical scheme of Parylene-C. (B) Biocompatible Parylene-C membranes maintaining hES cell-derived RPE. The Parylene-C membranes were constructed of 6.0 μm thick mesh work, which supported an array of an ultrathin top layer having a 0.3 μm thickness.³ (C) Schematic explanation of the cross-section of hES cell-derived RPE cells maintained on a Parylene-C membrane.³ (D) (a) Optical coherence tomography (right) and infrared image (left) of the hES cell-derived RPE maintained on a Parylene-C membrane under the retina. (b) hESC-derived RPE cells maintained on a Parylene-C membrane stained with hematoxylin and eosin, while exhibiting the subretinal positioning of the Parylene-C membrane in which the pigmented hES cell-derived RPE (arrow) was artificially removed from the Parylene-C membrane (arrow) in this case. The Parylene-C membrane is described as ultrathin sites (bracket) and thin sites (bracket) where nutrition migrates more easily.² Parts B-C adapted from ref. 3 with permission from Mary Ann Liebert, Inc., Copyright 2016. Part D adapted from ref. 2 with permission from Elsevier, Copyright 2015.

maintained on Parylene-C films was prepared in which Parylene-C films were formed by lithography (Figure 6.11B).³ The Parylene-C films were composed of a thick Parylene-C layer (6 μm) that supported an ultrathin region (0.3 μm). The final composition was a semipermeable and biostable support for the hES cell-derived RPE cells. hES cell-derived RPE cells on Parylene-C films formed an epithelial monolayer with tight intracellular junctions and became well-polarized with microvilli, which exhibited similar properties to RPE cells *in vivo*. The hES cell-derived RPE cells on Parylene-C films were implanted in the subretinal site of porcine or rat eyes (Figure 6.11C and D). hES cell-derived RPE cells on Parylene-C membranes could be alive for at least 12 months in immunocompromised animal models. The ultrathin Parylene-C film used in this research maintained hES cell-RPE cells as an artificial Bruch's membranes that allowed free permeation of water or small molecules through the polymeric film and provided a platform for hES cell-derived RPE cells to attain a differentiated and uniform monolayer.⁹⁶ Polarized monolayers of hES cell-derived RPE cells exhibited enhanced survival in comparison to cell suspension of hES

cell-derived RPE cells. Currently, human trials of the hES cell-derived RPE cells on Parylene-C films are ongoing (NCT02590692).

Stanzel and colleagues investigated a polarized monolayer of human adult stem cell-derived RPE cells cultured on PET, poly(ethylene terephthalate), films with a size of 1×1 mm with $10\text{-}\mu\text{m}$ thickness.¹⁰² An RPE monolayer on PET films were implanted subretinally in the rabbit eyes and was evaluated to have survived and retained their properties for more than 1 month in animal models. PET films, which have a pore diameter of $3.0\text{ }\mu\text{m}$ were used as a RPE monolayer supporter and resulted in much better preservation of the RPE layers in comparison to PET films having pore diameters of $0.4\text{ }\mu\text{m}$.¹⁰² Then, this investigation indicates that the porosity and pore size of the supporting artificial films of RPE cells are also key factors because the physical properties of the polymeric films affect the function and viability of RPE monolayers, which are cultured on the polymeric films.

Kamano and his colleagues reported a self-standing hiPS cell-RPE cell sheets using Transwell inserts. The timeline of the preparation protocol for the hiPS cell-RPE cell sheets is described in Figure 6.12A and B.¹⁵ First, hiPS cell-derived RPE cells were inoculated on Transwell inserts coated with collagen type I, where Transwell inserts were typically made of polyethylene terephthalate (PET) films with a pore size of $0.4\text{ }\mu\text{m}$. When hiPS cell-derived RPE cells became confluence on Transwell inserts, the PET films on the bottom of the Transwell inserts were released and the hiPS cell-derived RPE sheet composite on the Transwell inserts was digested with a collagenase from the bottom to dissolve the collagen gels (Figure 6.12C–E). Then, the hiPS cell-derived RPE sheet was detached from the Transwell inserts. The collagen gels and PET films of the Transwell inserts were used as temporal templates to create hiPS cell-derived RPE monolayer sheets (Figure 6.12F and G). The hiPS cell-derived RPE sheets showed expression of ECM (extracellular matrix) composition of the base membranes (laminin and collagen type IV) and ZO-1 (Figure 6.12H).^{15,84} The autologous hiPS cell-derived RPE cell sheet was implanted under the retina of one patient with wet AMD disease.^{15,84} The hiPS cell-derived RPE cell sheet, which was transplanted remained intact after one year from surgery. The visual acuity of the patient was unfortunately not enhanced, but was also not worsened as a side effect of the implantation of hADS cells, as reported in the literature.¹⁰³ No improvement in the visual acuity of the patient after the transplantation of the hiPS cell-derived RPE cell sheet should be considered to improve the transplantation method of the hiPS cell- and hES cell-derived RPE. One method to improve the transplantation method of the hPS cell-derived RPE cell sheet would be multiple cell sheets transplantation under the retina of the patients. It is further important to analyze the difference in the visual acuity enhancement by the implantation protocols using both cell sheet implantation and single cell injection. Schwartz and colleagues successively found that the patients implanted with single cell suspension of hES cell-derived RPE cells recovered their visual acuity,^{13,14} while transplantation of the hiPS cell-derived RPE cell sheet into patient did not improve their visual acuity, as reported.^{15,84}

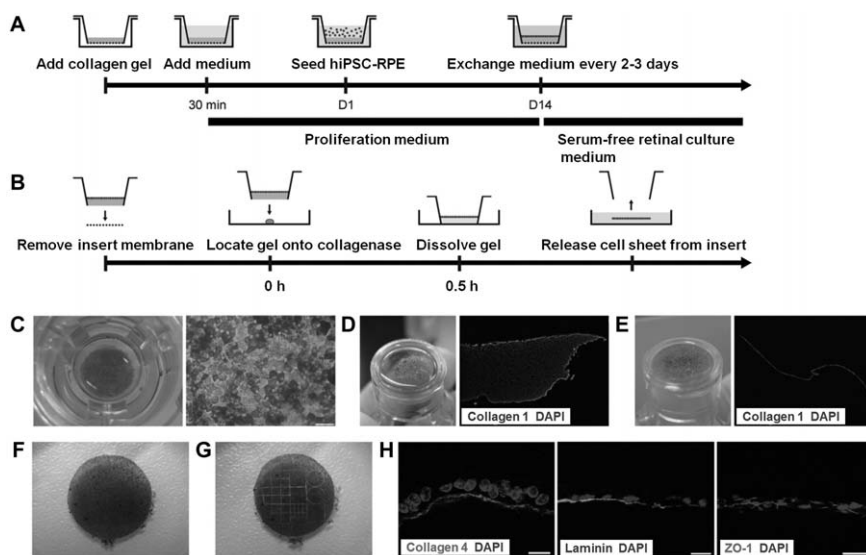


Figure 6.12 Preparation of hiPS cell-derived RPE and hiPS cell-RPE cell sheets. (A) The processing protocol of production of hiPS cell-derived RPE on the collagen gels in the cell culture insert. (B) The processing protocol of production of hiPS cell-derived RPE cell sheets. (C) Microscopic picture of the cell culture insert (left) and phase-contrast picture of hiPS cell-derived RPE cell sheets on the collagen gels. The bar indicates 50 μm . (D) Microscopic picture of the cell culture insert after the insert membrane was removed (left). Immunostaining of collagen type I on hiPS cell-derived RPE cultivated on the collagen gels before treatment with collagenase (right). (E) Low magnification of the cell culture insert after treatment with collagenase (left panel). Immunostaining of collagen type I on hiPS cell-derived RPE cultivated on the collagen gels after treatment with collagenase (right). (F) Low magnification image of a released hiPS cell-derived RPE cell sheet. (G) Grafts of cell sheets made by cutting the cell sheet using a laser microdissection system. (H) Immunostaining of basement membrane (laminin and collagen type IV) and tight junctions (ZO-1) in the cell sheet grafts.¹⁴ The bar indicates 20 μm .

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Developing biomaterials used for the support of the RPE layer with the appropriate porosity, pore size, morphology, and chemical structure as well as biocompatibility should be an interesting research topic for the successive implantation of stem cell-derived RPE cells in the therapies of macular degeneration diseases from a bioengineering perspective. Moreover, the extensive development of implantation equipment, for example a cell sheet shooter⁹⁴ that delivers RPE cell sheets into a subretinal region of the patients smoothly, should also be valuable from the bioengineering perspective for the transplantation of RPE cell sheets.

6.3.4 Bioengineering for Clinical Trials Using hES Cell-derived RPE Cells

Schwartz and colleagues investigated the first clinical trial of hES cell-derived RPE cells in several patients with dry AMD and Stargardt's macular dystrophy by subretinal implantation (NCT01344993 and NCT01345006).^{13,14} In this study, hES cells were exposed to animal cells (MEF: mouse embryonic fibroblast) during cell cultivation. Then, hES cell-differentiated RPE cells were especially analyzed for contamination of human- and animal-derived microorganisms.¹³ The hES cell-derived RPE cells were alive for the lifetime of the mice and integrated into the RPE layers of mice (Figure 6.13A), which were identified by immunostaining and expressed bestrophin in a basolateral style.¹³

The subretinal transplantation of hES cell-derived RPE was performed in nine patients with AMD and nine patients with Stargardt's macular dystrophy where three doses of RPEs (50 000, 100 000, and 150 000 cells) were used in patients who had each eye disorders.¹⁴ Figure 6.13B shows fundus images of eyes with pigmentation after transplantation with hES cell-derived RPE for the patients with AMD and Stargardt's macular dystrophy.¹⁴

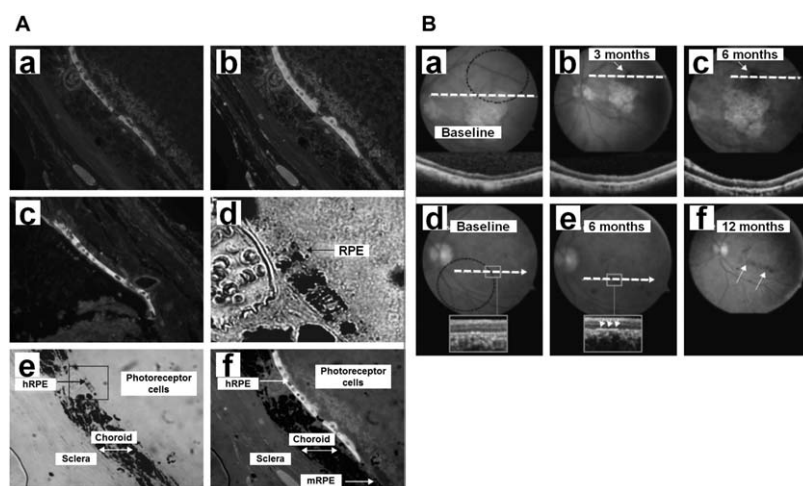


Figure 6.13 Implantation of hES cell-derived RPE into eyes. (A) (a–c) Immunohistochemical staining of hES cell-derived RPE with human mitochondria (a) and human bestrophin (b) with a merged image (c). (d and e) The images of the eye where the image of (d) is the enhanced image of (e). (f) The merged image of (a), (b), (c), and (e).¹³ Adapted from ref. 13 with permission from Elsevier, Copyright 2015. (B) (a–c) Color fundus images and spectral domain optical coherence tomography (SD-OCT) images of an eye of a patient with AMD where the dotted line describes an outline of the implanted region of hES cell-derived RPE at 0 (a), 90 days (b), and 180 days (c). The inset images show OCT imaging. (d–f) Color fundus and SD-OCT images of an eye of a patient with Stargardt's MD at 0 (d), 180 days (e), and one year (f).¹⁴ Adapted from ref. 14 with permission from Elsevier, Copyright 2015.

The pigmented patch of implanted cells became bigger and more pigmented half year after implantation in the AMD patients (Figure 6.13B(b) and B(c)) and 12 months after transplantation in the Stargardt's macular dystrophy patients (Figure 6.13B(e) and B(f)).¹⁴ Optical coherence tomography (inset) indicated the existence of RPE cells on the inner site of Bruch's membrane at half year in comparison to baseline (Figure 6.13B(a)–B(c)). Patches of pigmented cells were observed near the border of the baseline atrophy in the RPE cells (Figure 6.13B(e)) that were going to be more prominent at 12 months (Figure 6.13B(f), arrow) in the Stargardt's macular dystrophy patients.¹⁴ There was no evidence of systemic safety issues, adverse proliferation, or rejection generated by the implanted RPEs. Seventy-two percent of the patients were found to have patches of increased subretinal pigmentation consistent with implanted RPE.

The visual acuity was improved in ten patients, improved or remained the same in seven patients, and decreased in one patient, whereas the untreated eyes did not show any improvement in visual acuity (Figure 6.14).¹⁴ Furthermore, the peripheral and general vision were found to be increased in patients with both AMD and Stargardt's macular dystrophy.¹⁴ Their results suggest that hES cell-derived RPE provides a new source of cells for the treatment of patients with AMD or Stargardt's macular dystrophy.

Song and his colleagues investigated subretinal infusion of hES cell-derived RPE cells in four Asian patients (two patients with AMD and two with Stargardt's macular dystrophy (NCT01625559 and NCT01674829)).⁸³ No serious safety issues were found that were related to the transplanted RPE cells. Visual acuity was found to increase (recover) the identification of 9–19 letters in the Early Treatment Diabetic Retinopathy Study (ETDRS) chart for three patients and remained the same (+1 letter) for one patient.⁸³ This study also suggests that hES cell-derived RPE is useful for the treatment of macular diseases, such as AMD and Stargardt's macular dystrophy.

Several institutes are now engaging in stem cell therapies for patients with macular diseases using hES cell-derived RPE (Table 6.2). On the other hand, the output of these clinical trials has not yet been issued with the exception of the data discussed above at this moment.

6.3.5 Bioengineering for Clinical Trials Using hiPS Cell-derived RPE Sheets

Prof. Takahashi's group at Riken performed transplantation of hiPS cell-derived RPE sheets into patients with AMD. The hiPS cell-derived RPE sheets without supporting polymeric film was made as indicated in Figure 6.12.^{15,84} The first hiPS cell-derived RPE monolayer sheet was implanted into an old female (77 years old) with AMD in 2014.⁸⁴

The neovascular membrane was detached by surgical procedures and an autologous hiPS cell-derived RPE cell sheet (1.3×3.0 mm) was implanted under the retina with the use of a 1-ml syringe with a 20-gauge intravenous cannula attached (Figure 6.15).⁸⁴ The patient received no immunosuppressive

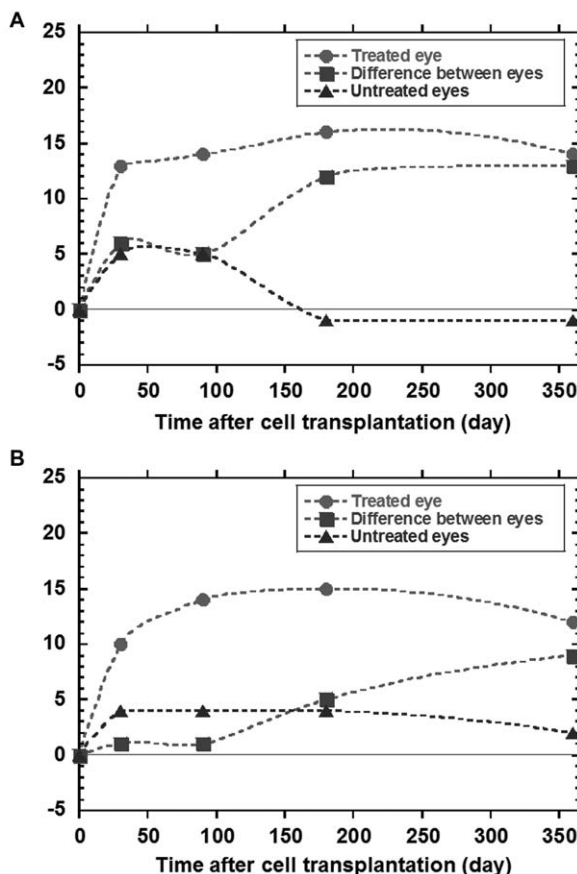


Figure 6.14 The improvement in visual acuity in patients with AMD and Stargardt's macular dystrophy after hESC-derived RPE transplantation. (A) Median change in patients with AMD in best-corrected visual acuity. (B) Median change in patients with Stargardt's macular dystrophy in best-corrected visual acuity. Top lines indicate the visual acuity change of eyes transplanted with hESC-derived RPE. The bottom lines indicate the untreated eyes. The middle lines describe the difference between the untreated and treated eyes of the patients.¹⁴ Adapted from ref. 14 with permission from Elsevier, Copyright 2015.

medicine after the implantation of the autologous hiPS cell-derived RPE cell sheet. The cell sheet was initially curled on the margin after implantation of the cell sheet but was gradually flattened by 8 weeks.⁸⁴ At one year after surgery, no sign of graft rejection or recurrence of the neovascular membrane was present. Good retinal integrity over the graft was observed at 12 months after the implantation.⁸⁴ Then, the implanted cell sheet was found to remain intact. On the other hand, the corrected visual acuity of the patient showed no improvement, but was maintained. Tumorigenicity and genomic aberrations of hiPS cell-derived RPE cell sheet were not observed in this patient.⁸⁴ It was

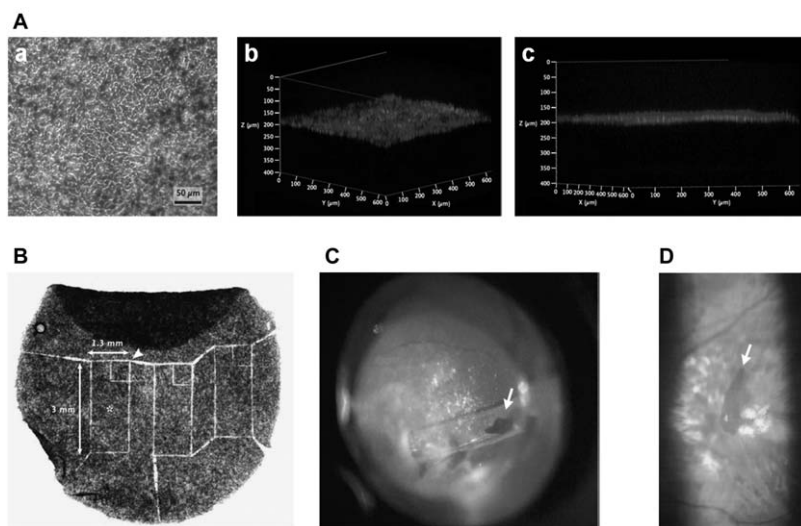


Figure 6.15 Generation and Transplantation of the iPS cell-RPE Sheet. Panel A (the three upper images) shows that the cell morphologic characteristics of the iPS cell-RPE sheet (*i.e.*, sheet of retinal pigment epithelial [RPE] cells differentiated from iPS cells) used for implantation, which shows a monolayered structure according to Z-stack images (middle and right), is consistent with typical RPE cell morphologic characteristics (left). Panel B exhibits the iPS cell-RPE sheet used for implantation. The graft sheet (asterisk) was 1.3×3.0 mm in size and had a cut in the corner (arrowhead) to distinguish its back and front sides. Panel C exhibits the iPS cell-RPE sheet (arrow) that was implanted under the fovea using a 1 ml syringe with a 20-gauge intravenous cannula attached. Panel D shows that the implanted iPS cell-RPE sheet (arrow) was readily visible on the day after surgery.⁸⁴ Adapted from ref. 84, <http://dx.doi.org/10.1056/NEJMoa1608368>, under the terms of the CC BY 4.0 license, <https://creativecommons.org/licenses/by/4.0/>.

summarized that the implantation of autologous hiPS cell-derived RPE cell sheet was feasible and safe for the treatment of patients with wet AMD in this study. Currently, the use of hiPS cell-derived cells for clinical trials is still limited. This limitation occurs because hiPS cells are generally generated by the transduction of genes into the cells using a virus vector, although there are several methods to create hiPS cells using non-integrated gene delivery and other protocols.¹²

6.3.6 Bioengineering for Clinical Trials Using Adult Stem Cells

Some human stem cells except pluripotent stem cells (hES cells and hiPS cells) have also been used instead of hPS cell-derived RPE cells, *e.g.*, hADS cells, hBMS cells, umbilical cord stem cells, neural stem cells from human

fetal forebrain tissues, and RPE stem cells taken from donor eyes.^{103–108} This chapter discusses the human trials of adult stem cell implantation for macular degeneration from a bioengineering perspective. These human trials are described in Table 6.3.

BM-derived CD34⁺ cells (endothelial progenitor cells and hematopoietic progenitor stem cells) have been especially used for BM implantation to reconstitute the hematopoietic system (0.4–1.0 mL of BM is generally used).¹⁰⁴ CD34⁺ cells have been implanted in animal models as potential therapies for the administration of degenerative and ischemic retinal diseases because of the multipotent properties of CD34⁺ cells and their trophic effects.¹⁰⁶ Intravitreally implanted CD34⁺ cells can migrate into the retina and home into the damaged neuronal tissues and retinal vasculature. The human CD34⁺ cells are found in the mouse retina even half year after implantation of CD34⁺ cells without related safety issues.¹⁰⁶ Then, Park and colleagues performed a pilot clinical trial (NCT01736059) where the safety and feasibility of the intravitreal implantation of autologous CD34⁺ cells were studied in the cure of patient eyes with ischemic and degenerative retinal diseases.¹⁰⁴ CD34⁺ cells were taken from 40–50 mL of BM that was suctioned from the patient's iliac crest. The mononuclear cell fraction (hBMN cells) was isolated by Ficoll density gradient method, which was followed by MACS method using CD34⁺ antibody. 3.4 million of CD34⁺ cells were intravitreally injected into patient eyes. The ophthalmic examination of optical coherence tomography (OCT) suggested no worsening of eye conditions during follow-up period except the patient eye of AMD who had mild progression of geographic atrophy in both the operated eyes and contralateral eyes. The new cellular incorporation into the macular of the hereditary macular degeneration was observed from the examination using adaptive optics OCT.¹⁰⁴ Although hES cell-derived RPE implantation is more powerful for the administration of AMD patients, the implantation of BM-derived CD34⁺ cells is simple to operate and might be feasible for the cure of eyes with ischemic or degenerative retinal diseases.

Although the side effect of implantation of hBMN cells and hBMS cells into the patients' eyes has not yet been reported, it should be mentioned that there could exist some side effect of stem cell therapy. Recently, Kuriyan investigated a negative effect of the implantation of undifferentiated and autologous hADS cells into patients with dry AMD intravitreally (NCT02024269).¹⁰³ Three patients were found to be blind (no light perception) by the implantation of autologous and undifferentiated hADS cells, which have been increasingly used by “stem cell clinics” in the USA and other countries to treat a variety of disorders. The complications that were found in the patients were considered to be created by the stem cell preparations rather than the implantation processes. This consideration occurred because ophthalmologists had experience using many intravitreal injections of other medications for many retinal diseases with no same complications.¹⁰³ The retinal detachments with severe proliferative vitreoretinopathy were found in three patients in this trial, although the patients

were undergoing observation after the implantation of hADS cells. Retinal detachment is 100% in this trial, which led to visual loss of the patients, while retinal detachment rates were found to be 15% in a phase I-IIa trial and 29% in a phase I trial of subretinal human umbilical tissue-derived cells.¹⁰³ On the other hand, the rate of retinal detachment after intravitreal administration of anti-VEGF was found to be less than 1%.¹⁰³ It is now important to systematically discuss the negative effects (side effects) of stem cell therapies, particularly for patients with macular degeneration.

One of the explanations of the side effects that originated from hADS cell transplantation could be because of the contamination of the remaining enzymes in the hADS cell solution, as the retinal detachments in the patient eyes should be a degradation of ECMs on the retina. In general, hADS cells are prepared by collagenase digestion of adipose (fat) tissues to generate single cell solution, while hBMN cells and hBMS cells can be obtained from a BM solution where there is no necessary step to using the collagenase digestion process. The collagenase digests one of the ECMs of the collagens. The residual collagenase and/or trypsin in the hADS cell solution, considered as contaminants, should be one of the candidates to explain the side effects originated by hADS cell transplantation, in which trypsin is used for the cell passage to digest ECMs during the cultivation of the fat tissues and BM solution on tissue culture flasks to purify the stem cells (hADS cells and hBMS cells) from the cell solution (fat tissue solution and BM solution, respectively). It is suggested that the bioengineering process in the preparation of the stem cells without contamination is a critical issue for the successful transplantation of the stem cells into the patients' eyes for the treatment of macular degeneration diseases.

6.3.7 Clinical Trials Using Fetal Stem Cells

Human central nervous system stem cells (hCNS-SCs) are possible to be purified from human brain tissues. hCNS-SCs are found to have the abilities to protect the host neurons in animal models of neurological diseases.¹⁰⁹ hCNS-SCs showed the potential to maintain hippocampal neurons in a mouse model of specific lysosomal storage disease. This study was followed by a clinical trial (Phase I, NCT00337636) for the cure of patients with neuronal ceroid lipofuscinosis, which is a fatal and severe neurodegenerative disorder.¹¹⁰ The clinical trials using hCNS-SCs (NCT02467634, NCT01632527, and NCT02137915) are ongoing in addition to some trials including those for the treatment of patients with AMD (Table 6.3). However, it should be considered that it is relatively difficult to get human fetal brain tissues for the purification of hCNS-SCs to be used in clinical trials, from a bioengineering perspective.

McGill and his colleagues prepared hCNS-SCs from second trimester human brain tissue.¹¹⁰ They demonstrated efficacy to protect photoreceptors and to preserve visual function by subretinal transplantation of hCNS-SCs into the Royal College of Surgeons (RCS) rats. RCS rats have a retinal abnormality,

which initiates at three weeks postnatal (P21) where RPE cannot perform phagocytosis of shed photoreceptor cells and then, apoptosis of photoreceptors starts, which is similar to human retinal degeneration.¹¹¹ Then, RCS rats are commonly used for animal models for the cure for macular degeneration disease. They examined viability and morphology of cone photoreceptors over time.⁸³ Photoreceptor cone arrestin, showing the entire photoreceptor cone profile, is visually observed. Non-dystrophic retinas of the control rats showed normal cone morphology and outer segment length, whereas loss of outer segments and highly disorganized cone morphology could be observed in the unoperated dystrophic retinas of RCS rats.⁸³ When hCNS-SCs were subretinally transplanted, photoreceptor cones were normal in appearance at postnatal day 40 and postnatal day 60. At postnatal day 90, the morphology of photoreceptor cones was still preserved with slight deformation. At postnatal day 240, some photoreceptor cones had disappeared and the surviving photoreceptor cones became shorter with smaller outer segments.⁸³

Most of hCNS-SCs transplanted into RCS rats expressed characteristic markers of neural stem/progenitor cells. There was no evidence of the differentiation of hCNS-SCs into RPE cells or photoreceptor cells. hCNS-SCs that were engrafted into RCS rats migrated in a radial pattern within the subretinal space. They reported no evidence of uncontrolled proliferation or abnormal host reaction induced by hCNS-SCs, which suggested that hCNS-SCs were well tolerated in the subretinal space.⁸³

Human fetal stem cells, for example, Wharton's jelly-derived mesenchymal stem cells (hWJ-MS cells), have some merits over other kinds of MS cells, such as the maintenance of their stemness for some passages and their high expansion rate.¹¹² hWJ-MS cells occur only a little immune response because of no expression of MHC II molecules and low expression of MHC I molecules and, hWJ-MS cells can be considered to be suitable cell source for allogeneic cell implantation.¹¹³ hWJ-MS cells are reported to induce differentiation into neurons, *e.g.*, retinal progenitor cells.¹¹⁴ Then, Leow and his colleagues studied the safety and efficacy of hWJ-MS cells on retinal function and structure of RCS rats where hWJ-MS cells were transplanted into the subretinal space of the eye with 2 μ l hWJ-MS cells (1×10^5 cells per μ l) at postnatal day 21 (Figure 6.16).¹¹⁵ Micro-computed tomography (micro-CT) scans showed that hWJ-MS cells were localized in the subretinal space. The outer nuclear layer (ONL) in the treated RCS rats was found to be preserved, whereas the ONL was not preserved in the control group (Figure 6.16B).¹¹⁵ hWJ-MS cells (stem 121-expressing (stem 121⁺) cells) were found to differentiate into photoreceptors (rhodopsin⁺ cells), Muller (GFAP⁺) cells, and bipolar (PKC- α ⁺) cells from observation with confocal microscopy with no retinal tumor formation (Figure 6.16C),¹¹⁵ although no significant improvement in retinal function was observed in RCS rats transplanted with hWJ-MS cells in this study. The subretinal injection of hWJ-MS cells delayed the loss of the photoreceptors in RCS rats in this study. Currently, there are no clinical trials using hWJ-MS cells, although hWJ-MS cells might be an interesting source of stem cells for the treatment of macular degeneration.

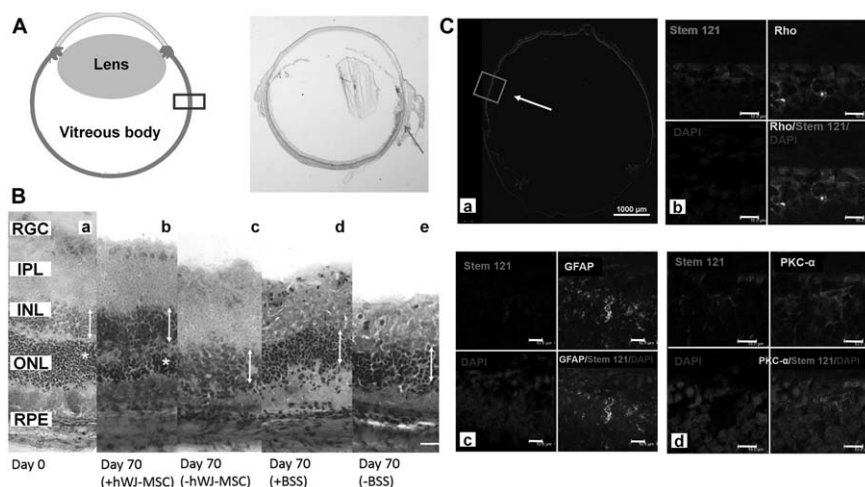


Figure 6.16 hWJ-MS cell injection into the subretinal region of the eye of RCS rats. (A) Schematic representation (left) and microscopy picture (right) of the eye where hWJ-MS cells were transplanted into the subretinal space of the eye of RCS rats. (B) Histology of the eyes of RCS rats where hWJ-MS cells were transplanted into the subretinal space of the eye (square in the left figure in (A)) at day 0 (a) and 70 (b–e). The ONL (asterisks) were observed in eyes transplanted with hWJ-MS cells (b) compared to thin layer of ONL in the eye without transplantation (c–e). The ONL could not be seen in the eye injected with BBS (borate buffered saline) (d) or without BBS (e) where INL indicates inner nuclear layer (double-headed arrows), IPL is the inner plexiform layer, and RGC is the retinal ganglion cell. The bar indicates 20 μ m. (C) (a) Confocal microscopy of the whole eye injected with hWJ-MS cells. The arrow represents the site of hWJ-MS cells injection. (b–d) Confocal microscopy of the magnified pictures of the site of hWJ-MS cells injection (box in (a)) with co-localization of DAPI and stem 121 by PKC- α , GFAP, and rhodopsin.¹¹⁵ Adapted from ref. 115, <https://doi.org/10.1371/journal.pone.0128973>, under the terms of the CC BY 4.0 license, <https://creativecommons.org/licenses/by/4.0/>.

This is because it is rather difficult to collect umbilical cords, and only a small amount of Wharton's jelly is obtained from each umbilical cord.

Currently, there are not clinical trials, which use hWJ-MS cells for the administration of macular degeneration diseases, although hWJ-MS cells could be a more important source of stem cells in comparison to hCNS-SCs. This is due to the easy acquisition of hWJ-MS cells.

6.3.8 Future Perspectives of Stem Cell Therapy for Macular Degeneration Diseases

Mature differentiated cells (RPE) or progenitor cells (e.g., oligodendrocyte progenitors and cardiac progenitors) differentiated from hPS cells are used in current clinical trials using hPS cells. More sophisticated isolation

(purification) techniques and efficient cell differentiation protocols of adult stem cells (hBMN cells, hBMS cells, and hADS cells)^{61,90} or fetal stem cells (hWJ-MS cells and hCNS-SCs) should be developed for clinical usage of stem cells from the bioengineering perspective. It could be important to develop cell sorting dishes,^{88,89} where stem cell-derived cells are cultivated on appropriate materials, and the main targeted lineages of cells remain (*i.e.*, are sorted) on the biomaterials, which will be used in clinical application. Furthermore, the development of cell differentiation dishes is also important when stem cells that are cultured on specific biomaterials are guided to differentiate into a targeted lineage of cells. Currently, only a few implanted stem cells remain at the infused domain of the patients. The infusion method of the stem cells or stem cell-derived cells into the patients should also be improved using injectable scaffolds, hydrogels, or membranes with specific cells, where the supporting materials will promote an enhancement in the long-term survival rate of the implanted cells in the patients.

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CHAPTER 7

Conclusions and Future Perspective on Biomaterial Control of Therapeutic Stem Cells

7.1 Introduction

The focus of this book, *Biomaterial Control of Therapeutic Stem Cells*, is on stem cell culture and differentiation where stem cells are isolated, cultured, and differentiated using biomaterials. Furthermore, we discuss the current status of clinical trials using human pluripotent stem (hPS) cells as well as human adult and fetal stem cells.

7.2 Chapter 1

In Chapter 1, we discussed general stem cell definitions, various types of stem cells, and biomaterial control of stem cell fate of proliferation and differentiation.

7.3 Chapter 2

Chapter 2 discussed adult stem cell culture on extracellular matrices (ECMs) and natural biopolymers. It is known that ECM proteins can determine whether stem cells are going to multiply or undergo growth retardation, move or remain static, and thrive or undergo apoptotic death. As a result, the ECM proteins are key factors in reproducing the biological roles of stem cells *in vitro*, which assist stem cells to cause induction into different lineages of

the cells. ECM proteins are the major cell development ingredients used to regulate the expansion and differentiation of stem cells in developing medicine and tissue design, both *in vivo* and *in vitro*. This chapter discusses in detail the differentiation of stem cells developed on biomaterials prepared with ECM proteins and on the chemical and biological contact between ECMs and stem cells.

ECM proteins not only work as supporting biomaterials for stem cells but also support to control cellular functions, especially determination of stem cell fate.^{1,2} Moreover, ECM proteins can regulate signal transduction generated by various bioactive molecules, including growth factors.^{2,3} The morphologies of mesenchymal stem (MS) cells is controlled by regulating the attachment of cells to ECM proteins, and cell morphologies can, in turn, control cell induction. ECMs in scaffolds or culture dishes can regulate MS cell morphologies and differentiation with high efficiency, which generates several possibilities for the application of stem cells in tissue engineering.⁴

The interaction between MS cells and specific ECM proteins can regulate induction of MS cells into specific lineages. The most widely used ECM proteins that regulate induction of MS cells into desired lineages are shown in Figure 7.1.⁵ Fibronectin (FN) appears to promote the induction of MS cells into adipocytes.⁴ Laminin-5 (LN-5), vitronectin (VN), and collagen (COL) type I regulate MS cells into osteogenic induction.^{6–8} The binding of integrin

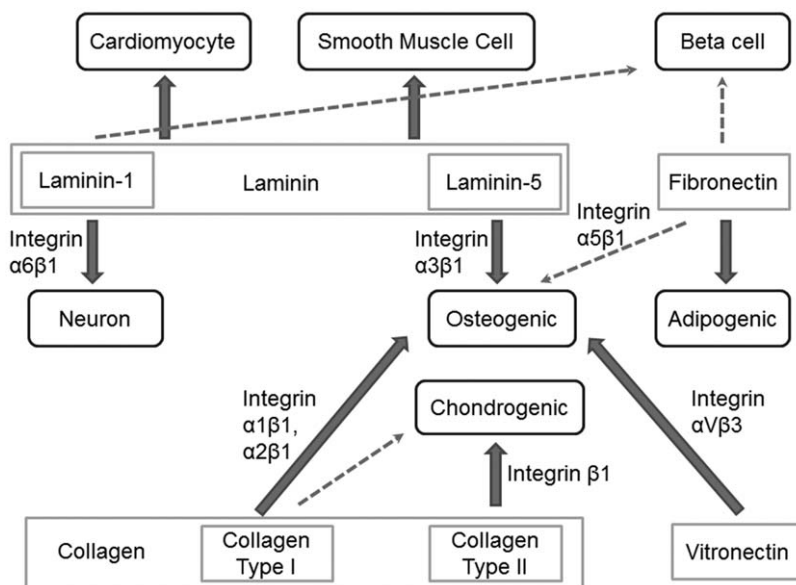


Figure 7.1 ECM proteins control stem cell fate of differentiation *via* integrin and non-integrin binding.³⁵
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receptors of MS cells is different, depending on the ECM protein. Laminin (LN) promotes differentiation of bone marrow stem (BMS) cells into smooth muscle cells and cardiomyocytes,^{9,10} whereas laminin-1 (LN-1) leads BMS cells into neural induction through integrin $\alpha_6\beta_1$.¹¹ Integrin $\alpha_v\beta_3$ mediates binding between VN and BMS cells.⁸ Integrin $\alpha_3\beta_1$ mediates the adhesion of BMS cells to LN-5,⁶ while integrin $\alpha_2\beta_1$ and $\alpha_1\beta_1$ mainly bind COL type I.^{7,8} The induction of BMS cells into β cells may be facilitated by interactions between MS cells and FN and/or LN-1.^{12,13}

Decellularized ECM scaffolds are attractive materials, as they can strongly retain the design of the original tissues and form biological environments more accurately than scaffolds made from single ECMs. Decellularized ECM scaffolds might be powerful tools for the induction of MS cells into several difficult lineages, such as hepatocytes, dopamine-secreting cells, and β cells.

Natural or synthetic biopolymers including ECM-oligopeptides are promising materials for scaffolds or hydrogels including MS cells. A variety of biomaterial designs for scaffolds and hydrogels immobilizing MS cells are possible using biopolymers that have ECM-oligopeptides, which allow cell attachment, expansion and induction into specific lineages. On the other hand, it is currently complicated to describe the direction of desired induction lineages from the interaction of MS cells and appropriate ECM-oligopeptides. The combination of base biopolymers and ECM-oligopeptides on scaffolds, as well as the physical and chemical properties of scaffolds, determine the induction of MS cells into desired lineages.

7.4 Chapter 3

In Chapter 3, we discussed recent advances in regards to the utilization of synthetic or natural materials and molecules to lead to the proliferation and differentiation of hPS cells, while supporting their pluripotent state in xeno-free and feeder-free cultivation.

The improvement in strategies for cultivation of these cells without using MEFs (mouse embryonic fibroblasts) as feeder layers contributes to more reproducible cultivation states and avoids the danger of xeno-origin contamination, thereby enhancing the expected clinical use of differentiated hPS cells. Human or recombinant VN, laminin-511, laminin-521, and FN, which are segments of ECMs, have been used rather than Matrigel for the feeder-free propagation of hPS cells. Promising hPS cell cultivation has been considered in the following conditions: in encapsulation within three-dimensional (3D) hydrogels made of alginate and/or other hydrophilic natural substrates, on microcarriers composed of synthetic polymeric materials, and on oligopeptide-immobilized surfaces derived from VN or LN.

hPS cells are commonly cultivated on human feeder cells or MEFs in cultivation media including KSR or in StemPro, mTeSR1, or another commercial cultivation medium used for feeder-dependent cultivation. After several passages, the hPS cells are transferred into a feeder-free cultivation condition using Geltrex- or Matrigel-coated plates with Essential 8, StemPro,

or mTeSR1 or other commercial cultivation medium. No types of cultivation system are preferable for clinical trials, because these cultivation media include some xenogenic bovine serum albumin (BSA) or because xeno-originated Matrigels, MEFs, or serum is used.

Some xeno-free and feeder-free cultivation methods have been recently developed; hPS cells can be cultivated on (1) PDL (poly-D-lysine)-coated plates in TeSR2 supplemented with Y27632, (2) StemAdhere-coated plates in TeSR2 media, (3) CELLstart (albumin and FN)-coated plates in StemPro, TeSR2, or another hPS cell cultivation medium; (4) Synthemax (a peptide derived from VN) plates in StemPro, TeSR2 media, or Essential 8 media; or (5) VN-coated plates in TeSR2 media or Essential 8 media; (6) LN-511 or LN-521-coated plates in TeSR2 media or Essential 8 media. However, not all hPS cells are able to expand and support their pluripotent state in each of the above serum-free cultivation conditions. Future establishment of coating biomaterials and cultivation matrices, including microfibers, nanofibers, microcarriers (MCs), hydrogels, and plates, is important for the long-term cultivation of hPS cells and for the establishment of appropriate hPS cell cultivation media.

Use of hPS cells in clinical therapy will demand hPS cell generation on a huge scale. Currently, hPS cell cultivation media and cultivation plates are extremely expensive and so it is difficult to generate large batches of hPS cells. Then, the surface architecture of microfibers and MCs for use in the mass preparation of hPS cells in bioreactor systems needs to be carefully designed. The present MC biomaterials used for hPS cell cultivation are restricted to crosslinked gelatin, dextran, and polystyrene beads. This is because the typical MCs used for hPS cell cultivation are commercially available beads. In two-dimensional (2D) cultivation, some excellent biomaterials are reported for the long-term cultivation of hPS cells; the biomaterials are completely synthetic plates and oligopeptide-immobilized plates under xeno-free and feeder layer-free cultivation systems. In this case, ECM-coated microfibers or MCs would not be appropriate for hPS cell proliferation due to the expense of using ECMs. Synthetic hydrogels, microfibers, nanofibers, or MCs, which maintain hPS cell expansion while keeping their pluripotent state, should be adequate in the future. It is important to prepare MCs having a polymeric (and surface) design that holds hPS cell proliferation and supports hPS cell pluripotency for long-term 2D cultivation. MCs that are immobilized with specific polymeric materials or immobilized with optimal peptides are considered to support many types of hPS cells by retaining the pluripotent state in a defined media for the long term (>15–30 passages).

At present, no meta-analysis has been carried out to evaluate why specific polymeric materials with a certain chemical structure maintain some hPS cell lines. Several specific polymeric materials, which were reported to maintain the pluripotency of hPS cells, still need to be re-evaluated using various cell lines and cultivation media, and the data need to be verified by other investigators. We do not know whether completely synthetic surfaces

can maintain pluripotency of hPS cells in xeno-free cultivation systems without a polysaccharide, oligopeptide, or ECM coating; indeed, typical synthetic biomaterials appear to need the production of ECMs from hPS cells or the sorption of proteins from a conditioned media of feeder cells (or other cultivation media) because of the heparin-like characteristics of biomaterials having a growth factor-adsorbed surface. Biological evaluation investigating synthetic biomaterials and surface coatings that maintain hPS cells would contribute to developing the most efficient synthetic material surface architecture or coating biomaterials for hPS cell cultivation.

7.5 Chapter 4

In Chapter 4, we discussed the physical cues of synthetic and natural polymeric materials that lead to the differentiation of hES cells and hiPS cells into several different lineages. Such lineages include dopamine-secreting neurons, neural cells, insulin-secreting β cells, hepatocytes, osteoblasts, chondrocytes, and cardiomyocytes. Recent studies have demonstrated that interactions between the physical microenvironments and the stem cells are critical in the establishment of stem cell differentiation. This chapter therefore examined physical cues from synthetic and natural materials, which helped to direct the differentiation of hiPS and hES cells into a variety of lineages. Particular focus is placed on how the fate of hPS cell differentiation is shaped by three factors, namely (1) the elasticity of materials chosen for hPS cell cultivation, (2) the topography of the materials used in this process and (3) the mechanical forces associated with the materials (electrical and stretching stimulation *via* materials). Cell morphology, focal adhesions, and cell phenotype can be affected by the elastic properties of materials for stem cell cultivation, which can control cell attachment. As cell functions are controlled by a complex topographical niche *in vivo*, including extracellular matrix geometry, nano- and micro-scale topographic surfaces guide stem cell differentiation fates.

hPS cells are unlimited and attractive cells, which are able to induce into any type of somatic cells in the human body and can expand infinitely. However, the diverse differentiation potential of hES and hiPS cells generates difficulty in regulating induction toward a greatly desired cell lineage, which could be used in regenerative medicine. Some outstanding induction procedures to regulate the induction of hPS cells into desired cell lineages have been reported. These methods do not contain the induction step that uses embryoid body (EB) generation,^{14–16} because EBs include many different kinds of induced cells even when their culture periods and sizes are well regulated.

The stiffness of materials in stem cell cultivation can control cell adherence and subsequently cell shape, cell phenotype, and focal adhesions, especially in 2D cultivation. The differentiation of hES and hiPS cells is sensitive to biomaterial stiffness, especially during early stages of induction. The optimal choice of cell cultivation materials of adequate stiffness can

enhance the efficiency of hES and hiPS cell induction into desired cell lineages, such as β cells, cardiomyocytes, dopamine-secreting cells, and retinal pigment epithelium.

A micro/nanograted (groove/ridge) patterned biomaterial of a specific width can facilitate the induction of hES and hiPS cells into β cells, cardiomyocytes, dopamine-secreting cells, and retinal pigment epithelium. Nanofibers retain a sophisticated 3D system for cultivation of hES and hiPS cells on nanopatterned biomaterials. hES and hiPS cells cultivated on aligned electrospun nanofibers can induce into β cells, cardiomyocytes, dopamine-secreting cells, and retinal pigment epithelium. Cyclic stretching of the materials where hES and hiPS cells are cultivated increases their induction into cardiomyocytes. Then, both electrical and mechanical stimulation of materials can contribute to enhancing hPS cell induction into desired cell lineages.

The control of stem cell induction into desired lineages is not yet completely understood. Cell cultivation biomaterials should be created with biomechanical, biochemical, and biophysical cues for this object. The development of materials requires multidisciplinary processes, which combine the choice of appropriate biomechanical stimulation, appropriate material morphology, adequate stiffness of biomaterials, appropriately ordered scaffold structures, and specific ECM proteins, and it will open up avenues to the controlled induction of stem cells into desired lineages.

7.6 Chapter 5

Chapter 5 discusses several protocols for inducing hPS cells cultivated on materials and considers the appropriate materials for hPS cell induction into targeted cell lineages. Human pluripotent stem cells (PS cells), including hiPS cells and hES cells, have the ability to induce differentiation into several cell types, which derive from the three germ layers for the disease treatment. On the other hand, it is challenging to control hPS cell differentiation into specific cell lineages because of their range of differentiation ability. An excellent strategy is to mimic the niche of stem cells for the differentiation of hPS cells into targeted lineages of the cells using appropriate polymers or natural materials for hPS cell cultivation. This chapter described various methods for inducing hPS cells cultivated on polymeric or natural materials and debated the optimal strategy and polymeric or natural materials for hPS cell induction into desired lineages of the cells. Recent trends in differentiation methods avoid EB generation because EBs include several kinds of differentiated cells.

In recent years, stem cell investigation has targeted the development of stem cells, which can be induced into desired lineages of cells, *e.g.*, functional cardiomyocytes, insulin-secreting cells, hepatocytes, and neurons. In these cases, the purity of the desired cell lineages was rarely investigated. Today, investigators are considering the use of stem cells for regenerative medicine and tissue engineering. For such a targeted goal for human stem

cells, it is necessary to ensure the purity of the induced cells. Moreover, the stem cell induction methods have been created under xeno-free cultivation systems. In this case, specific materials should contribute to the pluripotency of hES and hiPS cells and further regulate the differentiation fate of hES and hiPS cells.

At present, some sophisticated protocols have been reported for induction into appropriate lineages of cells, including cardiomyocytes,^{17,18} dopamine-secreting TH⁺ cells,¹⁶ and insulin-secreting β cells.^{14,15} On the other hand, it is still complicated to analyze which method is highly efficient and reliable for the induction of hPS cells in desired cell lineages. In particular, it is not known which cell cultivation materials are adequate for inducing differentiation into the specific cell lineages where the biomaterials are being created by different groups of investigators. One of the solutions to the complexity of evaluation of each cell culture material is that each investigator should report the expression of adequate and standardized induction markers on the induced cells that are able to be evaluated by flow cytometry. Our suggestions for induction markers are described in Table 7.1,¹⁹ and include major histocompatibility complex (MHC) for myoblasts, HNF4- α and ASGPR1 for hepatocytes, PDX1/NKX6.1 for pancreatic progenitor cells, TH for dopamine-secreting TH⁺ cells, cTnT for cardiomyocytes, and MAFA for mature β cells, which can be evaluated from flow cytometry using appropriate antibodies.

One of the recent methods of proliferation of hPS cells is spheroid cultivation. This is because it is not difficult to prepare large amounts of expanded hPS cells and the spheroids can be directly transferred to EB cultivation in an induction medium for differentiation of hPS cells. On the other hand, it should be noted that the purity of the induced cells into desired lineages of the cells through EB generation is not as high compared to the purity of the induced cells cultivated on 2D materials.

Some excellent materials have been reported for the cultivation and differentiation of hPS cells in 2D cultivation conditions. On the other hand, it is not easy to prepare a large amount of hPS cell-derived cells in a desired

Table 7.1 Suggested markers for the cells differentiated from hPS cells to analyze the purity of differentiated cells.¹⁹
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Differentiated cells	Markers
Mature β cells	MAFA, G6PC2
Pancreatic progenitor cells	PDX1/NKX6.1
Cardiomyocytes	cTnT
Myoblasts, myocytes	MHC (myosin heavy chain)
Dopaminergic neuron	TH (tyrosine hydroxylase)
Endothelial cells	CD31, vWF
Mature hepatocytes	ASGPR1, HNF4- α (AFP, albumin)
Retinal progenitor cells	CRX (cone-rod homeobox)

lineage in 2D cultivation conditions. hPS cell cultivation and induction on microcarriers in bioreactor systems indicates a realistic system for production of induced cells for clinical therapies; 10^6 – 10^{10} cells are expected to be used in clinical therapies.²⁰ On the other hand, the previously investigated microcarrier biomaterials are simple materials, such as gelatin (GEL), COL or dextran, which are not the best for the induction of hPS cells into desired cell lineages on microcarriers. It is important to design microcarrier grafting or coating biomaterials or to prepare microcarrier biomaterials for the optimal and efficient induction of hPS cells on the microcarriers.

The present hPS cell cultivation procedures use a batch-type process that uses disposable cell cultivation microcarriers or dishes. As a result, this process is laborious and expensive. A continuous cell cultivation process for the proliferation of hPS cells has been developed on thermoresponsive nanobrush interfaces.²¹ This system provides the microcarriers or plates immobilized on thermoresponsive nanosegments for the induction of hPS cells into desired cell lineages; the cells can be continuously harvested in the cultivation media by decreasing the temperature of the cultivation media, which would greatly reduce the cost of hPS cell cultivation and induction.

Currently, the clinical application of hPS cells is greatly restricted.²² On the other hand, ocular diseases are the first human trials of hPS cells and Phase I/II trials have exhibited promising safety results with some possible efficacy.^{23,24} The current clinical trials for hPS cell-based therapies are mainly concentrated on cure of retinal degeneration in the eye. This is due to the fact that eye tissue has an immunoprivileged property (tolerating properties of non-histocompatible cells and foreign antigens; extremely low immune response to foreign materials).²² Moreover, it is possible to see the internal tissues through a lens after implantation of the cells. Stargardt macular dystrophy and AMD (age-related macular degeneration) are the progressive degradation of light-sensing photoreceptors and their supportive RPE (retinal pigment epithelium).^{23,24} Some clinical trials using RPEs derived from hPS cells are now ongoing for Stargardt macular dystrophy and AMD.²² The extremely pure GMP (good manufacturing practice)-graded induced cells from hPS cells that are prepared on optimal materials would contribute to the cure of these ocular diseases and other disease treatments.

The preparation of materials for hPS cell induction under xeno-free systems would be one of the most important outcomes in biomedical investigation for the positive shift from hPS cell study to translational medicine. The combination of cell cultivation materials and optimal induction methods for the induction of hPS cells into desired cell lineages would produce a large quantity of extremely pure GMP-grade induced cells for use in tissue engineering and regenerative medicine.

7.7 Chapter 6

In Chapter 6, we discussed the current situation with stem cell therapy using hPS cells for patients with myocardial infarction (MI) and macular

degeneration, considering the bioengineering points of the therapy. Moreover, we considered clinical trials using adult or human fetal stem cells such as human mesenchymal stem (hMS) cells that were prepared to cure patients with these diseases.

Mature differentiated cells (RPE) or progenitor cells (*e.g.*, oligodendrocyte progenitors and cardiac progenitors) from hPS cells are used in current clinical trials using hPS cells. In the most fundamental research, hPS cells are induced to differentiate into mature stages of desired lineages of cells under xeno-free conditions. More sophisticated isolation (purification) techniques and efficient hPS cell differentiation protocols of adult stem cells [hBMN (human bone marrow mononuclear) cells, hBMS (human bone marrow stem) cells, and hADS (human adipose-derived stem cells)^{25,26} or fetal stem cells (hWJ-MS, human Wharton's jelly-derived mesenchymal stem) cells and hCNS-SCs (human central nervous system stem cells)] should be developed for clinical usage of stem cells from the bioengineering perspective. It is increasingly important to discuss in detail and systematically the negative effects (side effects) of stem cell therapies for patients with macular degeneration. The goal of this chapter is that readers, especially bioengineering investigators, will consider closing the gaps between fundamental research and clinical therapies of stem cells. It may be important to develop cell sorting dishes,^{27,28} where stem cell-derived cells are cultivated on appropriate materials, and the main targeted lineages of cells remain (*i.e.*, are sorted) on the biomaterials, which will be used in clinical application. Furthermore, the development of cell differentiation dishes is also important when stem cells that are cultured on specific biomaterials are guided to differentiate into a targeted lineage of cells. The development of simple and efficient differentiation and isolation protocols of stem cells using materials safely should be one of the priority tasks for bioengineering investigators who are involved in stem cell studies. Currently, only a few implanted stem cells remain at the infused domain of the patients. The infusion method of the stem cells or stem cell-derived cells into the patients should also be improved, using injectable scaffolds, hydrogels, or membranes with specific cells, where the supporting materials will promote an improvement in the long-term survival rate of the implanted cells in the patients.

Finally, the banking of hPS cell lines that are clinically approved, is necessary to provide a source of hPS cells as well as differentiated cells derived from hPS cells. It is also necessary to develop immune-compatible hPS cell lines for disease treatment, in addition to the excellent methods of induction of hPS cells into desired cell lineages. It is reported that hES cells show expression of low levels of MHC proteins and have immune-privileged characteristics.^{29–31} Taylor and his colleagues noted that the development of about 150 hES cell lines would be enough to provide sufficient HLA (human leukocyte antigen) to match hES cell derivatives for most patients in the UK.^{32,33}

Moreover, Nakajima and his colleagues suggested that 170 hES cell lines would cover the HLA matching of 80% of patients in Japan.³⁴ In another

issue, there are ethical concerns about the use of hES cells because of the destruction of human fertilized eggs, while typical and current protocols of hiPS cell generation use gene transduction *via* viral vectors, which may create modification of human genes in the cells. A reliable hiPS cell preparation protocol with excellent efficiency through a perfectly non-integrated gene transduction protocol or by using non-genetic molecules such as epigenetic-modifying small molecules³⁵ needs to be established for the use of differentiated cells derived from hPS cells, which are cultivated on optimal materials for future clinical use.³²

It will be challenging to control the differentiation fate of stem cells by regulating their microenvironment, such as by controlling only physical matrices or material characteristics in the stem cell niche, because biological cues are more effective in guiding the stem cell fate of differentiation. However, this issue also holds an important meaning in terms of human society; our performance and ability should be improved by our environment, which is not controlled solely by our heredity. We think that the role of the stem cell microenvironment in deciding and regulating stem cell fate of differentiation is similar to that of the human fate in our society.

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